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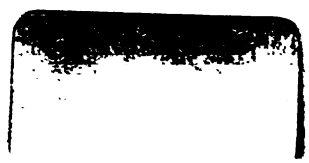

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K. H. a. P.

Dr. HERMANN O. L. FISCHER
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TORONTO 5, CANADA

MONOGRAPHS ON BIOCHEMISTRY

EDITED BY

R. H. A. PLIMMER, D.Sc.

AND

F. G. HOPKINS, M.A., M.B., D.Sc., F.R.S.

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THE CHEMICAL CONSTITUTION OF THE PROTEINS

BY

R. H. A. PLIMMER, D.Sc.

ASSISTANT PROFESSOR OF PHYSIOLOGICAL CHEMISTRY IN, AND FELLOW OF UNIVERSITY COLLEGE, LONDON

IN TWO PARTS

PART I

ANALYSIS

WITH DIAGRAMS

SECOND EDITION

LONGMANS, GREEN AND CO.

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TO

EMIL FISCHER

THE MASTER OF

ORGANIC CHEMISTRY IN ITS RELATION TO BIOLOGY

710

GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason, an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross-references, which are apt to be wrongly cited, and in order that each monograph may yield full and independent information of the work which has been done upon the subject.

It has been decided as a general scheme that the volumes first issued shall deal with the pure chemistry of physiological products and with certain general aspects of the subject. Subsequent monographs will be devoted to such questions as the chemistry of special tissues and particular aspects of metabolism. So the series, if continued, will proceed from physiological chemistry to what may be now more properly termed chemical physiology. This will depend upon the success which the first series achieves, and upon the divisions of the subject which may be of interest at the time.

R. H. A. P.
F. G. H.

710

PREFACE.

THE substance Protein, which constitutes the most important part of the material basis of all animal and vegetable life, has naturally attracted the attention and energy of numerous investigators throughout the past century. Progress in the study of this subject, on account of its difficulty, has been exceedingly slow, and it is only of recent years that the discovery of new methods by Emil Fischer has enabled us to increase our knowledge to its present extent. By these methods we have been able to advance from the conception of "albumin" to its actual separation into numerous units, and also to determine their arrangement in the molecule. On this account a monograph embodying the results of the most recent investigations, together with their connections with the work of the other and earlier investigators, needs no excuse for its appearance, as the subject is now being studied in every direction.

On account of the mass of material connected with the subject, this monograph has exceeded the proposed limit in length, and consequently it has become necessary to divide it into two parts :—

I. { The Chemical Composition of the Protein Molecule.
   { The Chemical Constitution of its Units.

II. The Synthesis of the Proteins.

R. H. A. P.

PREFACE TO SECOND EDITION.

THREE years have elapsed since the appearance of the first edition of this monograph. During these years there has been continuous work on the chemistry of the proteins. What is the result? No startling discovery has been made, but we have a better and deeper view of what is known. Emil Fischer, our master, has entrusted his ester method to his former pupil, Abderhalden, and has himself studied the more difficult question of the synthesis of the proteins (the polypeptides) and the peculiar optical properties of the amino acids. These properties, he thinks, give us an insight into chemical substitution in general.

Abderhalden has analysed, it would seem, as many proteins as possible, and has also devoted much labour to the polypeptides and the action of enzymes; in fact, the amount of work which he has accomplished is extraordinary. His analyses of the proteins are of great physiological interest, and are for the purpose of ascertaining whether proteins of similar origin are identical and whether they differ at different times of life. His chemical work on the polypeptides is intimately connected with the synthesis of the proteins and must lead later to a knowledge of those ill-defined substances, the proteoses and peptones. His physiological work on these compounds is concerned with the enzyme action of various tissues and the problem of nutrition.

Osborne is giving us as complete an analysis of his vegetable proteins as he has given us of their preparation and properties. From his careful and critical study of the data obtained by the ester method he comes to the conclusion that only about 5-15 per cent. of some of these proteins remains to be accounted for.

Our methods of analysis of the proteins have been extended and improved by Van Slyke, who, in conjunction with Levene, has also introduced a method for the investigation of what is termed the "leucine" fraction.

There were only a few gaps in the section devoted to the synthesis of the amino acids; one has been completely filled by Pyman, who has synthesised histidine.

In this new edition a more detailed account of the methods of analysis of the proteins has been given. The omissions of the previous edition which have been kindly pointed out to me have been inserted, and it is hoped that this edition will have a larger usefulness than the first.

My thanks are due to Mr. H. J. Page for his help in reading the proofs.

R. H. A. P.

January, 1912.

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THE CHEMICAL CONSTITUTION OF THE PROTEINS.

PART I.

INTRODUCTION.

THE proteins, of which we know some forty or fifty natural ones occurring in both animals and plants, are divided according to their origin, solubility, coagulability on heating and other physical characteristics into the following groups:—

I. Protamines, *e.g.*, salmine, sturine, clupeine, scombrine, cyclopterine, cyprinine.

II. Histones, *e.g.*, thymus histone, Lota histone, Gadus histone, histone from blood corpuscles.

III. Albumins, *e.g.*, ovalbumin, conalbumin, serum albumin, various plant albumins.

IV. Globulins, *e.g.*, serum globulin, fibrinogen and its derivative fibrin, myosinogen and its derivative myosin occurring in animals; legumin, conglutin, amandin occurring in plants, and some crystalline vegetable globulins, *e.g.* edestin, excelsin.

V. Glutelins, *e.g.*, glutenin in wheat, oryzenin in rice, soluble in very dilute alkali.

VI. Gliadins, *e.g.*, wheat-gliadin, hordein, zein, occurring in cereals and soluble in 70-80 per cent. alcohol.

VII. Phosphoproteins, *e.g.*, caseinogen, vitellin, ichthulin.

VIII. Scleroproteins, *e.g.*, keratin from hair, horn, feathers, egg-membrane. Collagen, gelatin, elastin. Silk-fibroin, silk-gelatin.

IX. Conjugated Proteins:—

(*a*) Nucleoproteins: nucleic acid in combination with protein generally I., II., III.

(*b*) Chromoproteins: chromogenic substance in combination with protein, *e.g.*, hæmoglobin.

(*c*) Glucoproteins: carbohydrate in combination with protein, *e.g.*, mucin, ovomucoid.

X. Derivatives of Proteins:—

(*a*) Metaproteins, *e.g.*, acid-albumin, alkali-globulin.
(*b*) Proteoses, *e.g.*, caseose, albumose, globulose.
(*c*) Peptones, *e.g.*, fibrinpeptone.
(*d*) Polypeptides, *e.g.*, glycyl-alanine, leucyl-glutamic acid, a tetrapeptide (2 glycine + 1 alanine + 1 tyrosine).

Except the protamines, the histones and the derivatives of the proteins, all the proteins contain carbon, hydrogen, nitrogen, sulphur, and oxygen, and they possess the following elementary composition:—

C	51-55	per cent.
H	7	,,
N	15-19	,,
S	0·4-2·5	,,
O	20-30	,,

from which a formula such as

$$C_{726}H_{1174}N_{194}S_3O_{214},$$

which is that of globin, the basis of hæmoglobin, can be calculated.

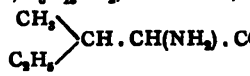
The phosphoproteins and the nucleoproteins contain also the element phosphorus; in the former, probably combined directly with one of the constituents of the protein molecule; in the latter, combined with a purine base or a carbohydrate, which substances constitute nucleic acid.

Investigations upon their chemical constitution have been carried on now for nearly a century, but it is only during the last ten years that, by the work of Emil Fischer and his pupils, any clear view has really been obtained of their actual constitution. The main result of these investigations is that the protein molecule is built up of a series of amino acids, which form the basis of their composition, and of which the following have been definitely determined:—

A. Monoaminomonocarboxylic acids.

1. Glycine, $C_2H_5NO_2$, or amino-acetic acid.
$CH_2 . (NH_2) . COOH$
2. Alanine, $C_3H_7NO_2$, or α-aminopropionic acid.
$CH_3 . CH(NH_2) . COOH$
3. Valine, $C_5H_{11}NO_2$, or α-aminoisovalerianic acid.
$(CH_3)_2 CH . CH(NH_2) . COOH$
4. Leucine, $C_6H_{13}NO_2$, or α-aminoisocaproic acid.
$(CH_3)_2 CH . CH_2 . CH(NH_2) . COOH$

5. Isoleucine, $C_6H_{13}NO_2$, or α-amino-β-methyl-β-ethyl-propionic acid.



6. Phenylalanine, $C_9H_{11}NO_2$, or β-phenyl-α-aminopropionic acid.

$C_6H_5 . CH_2 . CH(NH_2) . COOH$

7. Tyrosine, $C_9H_{11}NO_3$, or β-parahydroxyphenyl-α-aminopropionic acid.

$HO . C_6H_4 . CH_2 . CH(NH_2) . COOH$

8. Serine, $C_3H_7NO_3$, or β-hydroxy-α-aminopropionic acid.

$CH_2(OH) . CH(NH_2) . COOH$

9. Cystine, $C_6H_{12}N_2O_4S_2$, or dicysteine, or di-(β-thio-α-aminopropionic acid).

$HOOC . CH(NH_2) . CH_2 . S—S . CH_2 . CH(NH_2) . COOH$

B. Monoaminodicarboxylic acids.

10. Aspartic acid, $C_4H_7NO_4$, or aminosuccinic acid.

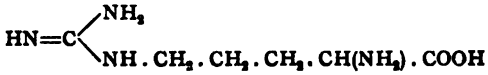
$HOOC . CH_2 . CH(NH_2) . COOH$

11. Glutamic acid, $C_5H_9NO_4$, or α-aminoglutaric acid.

$HOOC . CH_2 . CH_2 . CH(NH_2) . COOH$

C. Diaminomonocarboxylic acids.

12. Arginine, $C_6H_{14}N_4O_2$, or α-amino-δ-guanidinevalerianic acid.

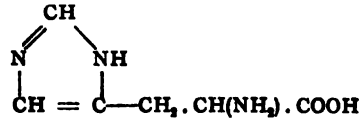


13. Lysine, $C_6H_{14}N_2O_2$, or α, ε-diaminocaproic acid.

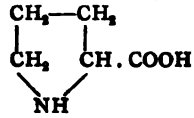
$H_2N . CH_2 . CH_2 . CH_2 . CH_2 . CH(NH_2) . COOH$

D. Heterocyclic compounds.

14. Histidine, $C_6H_9N_3O_2$, or β-imidazole-α-aminopropionic acid.



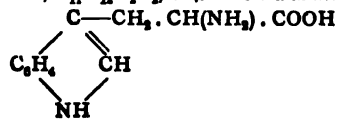
15. Proline, $C_5H_9NO_2$, or α-pyrrolidine carboxylic acid.



16. Oxyproline, or oxypyrrolidine carboxylic acid.

$C_5H_9NO_3$

17. Tryptophane, $C_{11}H_{12}N_2O_2$, or β-indole-α-aminopropionic acid.



E. Ammonia.

This long list is sufficient evidence of the complexity of the protein molecule; and, as yet, it seems to be incomplete, for several other products have been described. Of these, the presence of aminobutyric acid, which would complete the series of monoaminomonocarboxylic acids, was assumed by Schützenberger, but has not been demonstrated by any of the subsequent investigators. A large number of new products were added to the list by Skraup in 1904, but he has since shown that two of them were mixtures of glycine and alanine. Another amino-oxy acid was described, as also caseanic and caseinic acids; the latter is apparently identical with Fischer and Abderhalden's diaminotrioxydodecanic acid $C_{12}H_{26}N_2O_5$. Another product, diaminooxysebacic acid, was stated by Wohlgemuth to be a constituent, and a substance, $C_{11}H_{12}N_2O_3$, termed oxytryptophane was described by Abderhalden and Kempe; Gortner mentions the presence of a body in keratin which gives Millon's reaction, but is not tyrosine. Their presence as well as that of those described by Skraup has not been definitely proved; they cannot therefore be regarded as units of the protein molecule, and cannot be included in the above list until their presence in the molecule is thoroughly established.

3, 5-Diiodotyrosine is present in the protein contained in corals and other sea animals: it is formed when proteins are treated with the halogen: it is not included since it is of such rare occurrence and presumably a derivative of tyrosine.

The presence of glucosamine in the protein molecule is also a disputed question; there is no doubt that a carbohydrate containing nitrogen is contained in the glucoproteins in their prosthetic group, but it is doubtful if it be present in the protein part of the molecule, although a carbohydrate has been obtained from carefully purified proteins containing no prosthetic group, such as crystallised egg-albumin, serum-albumin (Langstein). The fact that the yield of carbohydrate from such a protein becomes smaller the more often it is recrystallised, suggests that the presumably pure protein still contained an impurity; this impurity would be a glucoprotein, which is found in both egg white and serum from which the crystallised proteins are separated, and this would give rise to the carbohydrate. Glucosamine is therefore excluded from the above list.

Numerous amino acids—including diamino- and oxyamino-acids—have also been synthesised of recent years by Neuberg and his co-workers and by Sörensen. Our knowledge of these acids should render the task of identifying a new unit in the protein molecule less laborious

than it has hitherto been. Their preparation was no doubt due to the possibility of the presence of other units than those above described, which possibility will not be excluded until the quantity of products isolated reaches 100 per cent.

The amino acids composing the protein molecule are generally referred to as the bricks, or foundation-stones, "Bau-steine"; but as the English translation of the German word is not entirely expressive of its meaning, it is preferable to use the term unit or element for these compounds in their relation to the proteins.

The methods which have been employed for the purpose of determining the composition of the protein molecule have been many and various. They may be classified under four headings:—

(1) Fusion with alkali.

(2) Oxidation with permanganate, chromic acid, etc.

(3) Action of halogens.

(4) Hydrolysis.

Of these, the last, that of hydrolysis, has thrown most light on the darkness of this complex problem. Hydrolysis has been effected by the action of acids, of alkalies, and of the various proteoclastic enzymes which occur in plants and animals, and is practically the only method by which we have attained to our present knowledge. Proteins were first hydrolysed by acids in 1820 by Braconnot, who used dilute sulphuric acid; between 1850 and 1875 hydrochloric acid was most frequently employed as the hydrolysing agent by Ritthausen, Hlasiwetz and Habermann and others, and from 1870 to 1880 Schützenberger employed baryta water under pressure. The action of vegetable enzymes on proteins has been studied chiefly by Schulze and his coworkers, that of animal enzymes by Kühne, Kossel, Kutscher, Drechsel, and numerous other investigators.

As the result of hydrolysis a complex mixture of all, or nearly all, the previously mentioned units is obtained. These have been isolated by various methods based upon the fractional crystallisation of the compounds themselves, or of their copper, silver, and other salts. Only when one or more of the amino acids occurred in somewhat large amounts was their isolation and characterisation effected; their amount seldom reached a value higher than 20 per cent. of the total quantity, and the remainder was represented by uncrystallisable syrups of unknown nature. The products described by Schützenberger have been shown by Hugounenq, Galimard and Morel to be mixtures of now definitely known substances.

A great advance was made when Drechsel discovered that the protein molecule contained diamino acids as well as monoamino acids, and to Kossel and Kutscher we owe our chief knowledge concerning their isolation and estimation. Emil Fischer, in 1901, by his study of the amino acids and their derivatives, introduced a new method of isolating and separating the monoamino acids, which depended upon the fractional distillation *in vacuo* of their esters, and which is now commonly known as the *ester method*. This method, though not yet really quantitative, has enabled us to obtain a knowledge of some 70 per cent. of the total products resulting by hydrolysis, and it has shown us that phenylalanine, serine, and alanine, which were only known to occur in a few, are present in all proteins, and that phenylalanine in its distribution is the principal aromatic constituent, for it often exceeds in amount that of tyrosine and occurs when this latter is absent. Further, it has demonstrated the presence of two new compounds, proline and oxyproline.

New units have thus been discovered with each improvement in, and development of, the methods of analysis of the proteins. The exact constitution of these units had also to be determined. This portion of the subject has been attended with entire success; with the exception of oxyproline we now know definitely the chemical constitution of every unit in the protein molecule mentioned in the above list. Considerable labour has been expended by investigators in proving the composition of the amino acids; thus, for example, tyrosine was discovered by Liebig in 1846 and its constitution established by synthesis in 1883 by Erlenmeyer and Lipp; serine was first isolated by Cramer in 1865 and synthesised in 1902 by Fischer and Leuchs; cystine was known in 1810 (Wollaston), was proved to be a constituent of proteins by Mörner in 1899, and its composition was established in 1903 by Erlenmeyer, jun. The latest addition to the list is histidine, which was first obtained by Kossel in 1896 and was synthesised by Pyman in 1911.

A further detail in our exact knowledge is still required. With the exception of glycine all the amino acids are optically active; the actual synthesis of the natural compound is only accomplished when the synthetic form has been separated into its isomers. A brief summary of the discovery and synthesis of the amino acids present in the protein molecule is given in Table A (p. 8). Several blank spaces still exist.

Mention must be made of the help which this nearly complete

chapter has afforded to the subjects of "alcoholic fermentation" and "ptomaines" or "natural amines". The amino acids have been found to be the mother substances of the by-products (fusel oil) of alcoholic fermentation and of those products of putrefaction—the amines—which are of biological importance. A description of these compounds is given in Harden's *Alcoholic Fermentation* and in Barger's *Simple Natural Bases*.

The final problem in the chemical constitution of the proteins—the synthesis—remains. This problem is still in its infancy. After numerous attempts by the earlier investigators its foundation was laid by Emil Fischer, who has synthesised a compound which, if it had been found in nature, would have been described as a protein. The difficulties in this part of the subject are very considerable. Not only is the amino acid required, but also its natural optical isomer is required. Many of the amino acids can only be readily obtained by decomposition of the protein, and even if they be prepared by synthesis much time and expense is involved. The proper conjunction of the amino acids is then necessary; the results of analysis give no clue as to whether the arrangement is a, b, c, d, e or b, c, a, d, e or d, e, c, a, b, etc.

The proper arrangement of the units in the molecule is under investigation. Several polypeptides, as the combinations together of the amino acids are termed, have now been prepared directly from the proteins; their isolation is so difficult that there must be many years of incessant labour before a real natural protein will be actually produced in the laboratory.

Like the complex proteins the polypeptides are hydrolysed into their constituent amino acids by the proteoclastic enzymes present in animals and plants. Some are hydrolysed by trypsin, others are not hydrolysed by this enzyme, but they may be hydrolysed by other enzymes. A complex polypeptide may be attacked at different junctions by different enzymes. Such data serve in determining the arrangement of the amino acids in the protein molecule, and they will no doubt help towards the elucidation of the nature of the enzymes themselves.

Use is also being made of the polypeptides in the study of disease.

The study of the Chemical Constitution of the Proteins can therefore be divided into four main sections:—

I. The Chemical Composition of the Protein Molecule.
II. The Chemical Constitution of the Units.
III. The Synthesis of the Proteins.
IV. The Action of Enzymes on the Polypeptides.

TABLE A.

	Discovered		Racemic "dl" Form Synthesised		Natural Active Form Synthesised		
	by	in	by	in		by	in
Glycine	Braconnot	1820	Perkin and Duppa	1858	—	—	—
Alanine	Schützenberger, Weyl	1888	Strecker	1850	d	Fischer	1899
Valine	v. Gorup-Besanez	1856	Fittig and Clark	1866	d	,,	1906
Leucine	Proust; Braconnot	1818; 1820	Limpricht; Schulze and Likiernik	1855; 1885	l	,,	1900
Isoleucine	F. Ehrlich	1903	Bouveault and Locquin	1905	d	Locquin	1907
Phenylalanine	Schulze and Barbieri	1881	Erlenmeyer and Lipp	1883	l	Fischer and Schöller	1907
Tyrosine	Liebig	1846	Erlenmeyer and Lipp	1883	l	Fischer	1900
Serine	Cramer	1865	Fischer and Leuchs	1902	l	Fischer and Jacobs	1906
Cystine	Wollaston; Mörner	1810; 1899	Erlenmeyer, jun.	1903	l	Fischer and Raske	1908
Aspartic Acid	Plisson	1827	Dessaignes	1850	l	Piutti	1887
Glutamic Acid	Ritthausen	1866	Wolff	1890	d	Fischer	1899
Ornithine	Jaffé	1877	Fischer	1900	d	Sörensen	1905
Arginine	Schulze and Steiger	1886	Schulze and Winterstein; Sörensen	1899; 1910	d	...	...
Lysine	Drechsel	1889	Fischer and Weigert	1902	d	...	...
Proline	Fischer	1901	Willstätter	1900	l	Fischer and Zemplen	1909
Oxyproline	,,	1902	Leuchs (?)	...	l	...	...
Histidine	Kossel	1896	Pyman	1911	l	Pyman	1911
Tryptophane	Hopkins and Cole	1901	Ellinger and Flamand	1907	l	...	...

SECTION I.

THE CHEMICAL COMPOSITION OF THE PROTEIN MOLECULE.

Hydrolysis.

IT has been already stated that the method of hydrolysis is the only one which has contributed to our present knowledge of the chemical composition of the proteins, and that the hydrolysis has been effected by (1) boiling with acids, (2) boiling with alkalies, (3) the action of proteoclastic enzymes.

Hydrolysis by the action of proteoclastic enzymes is never complete. The earlier investigators always observed that a complex body—anti-peptone—resistant to the further action of the enzyme was formed. Fischer and Abderhalden have confirmed this observation and have found that the resistant body contains all the phenylalanine and proline present in the protein molecule; even by the combined action of pepsin and trypsin, although phenylalanine and proline are formed under these conditions, a body resistant to enzyme hydrolysis still remained. Almost complete hydrolysis may be effected by trypsin and the enzymes in the small intestine if a sufficiently long time be given for the digestion. Hydrolysis by enzymes, though of use in the discovery of new units in the protein molecule, is not serviceable for a complete analysis of the decomposition products.

Hydrochloric acid, sulphuric acid, and hydrofluoric acid are the acids which have been used in hydrolysis, caustic soda and baryta the alkalies.

Several observers have maintained that the results obtained by hydrolysing with concentrated hydrochloric acid and 25-33 per cent. sulphuric acid are different, but recent investigations by Abderhalden and Funk, Skraup and Türk and others have shown that complete hydrolysis is effected by both acids, if the boiling be continued for a sufficiently long time. It is customary to boil with hydrochloric acid for six to twelve hours, with sulphuric acid for twelve to twenty hours, and to ascertain if the hydrolysis is complete by performing the biuret test on the solution and on the residue which always remains when the

hydrolysis is finished. Further hydrolysis is required if the biuret test is positive. Many proteins are hydrolysed with great slowness, as has been pointed out by Osborne and Jones who, by making use of Van Slyke's method for estimating amino groups, find that hydrolysis with concentrated hydrochloric may only be complete after two to five days. The length of time of hydrolysis is therefore of primary importance for a complete analysis.

Hugounenq, who has employed hydrofluoric acid, finds that the results depend on the strength of the acid; the stronger the acid the greater is the amount of complex polypeptides. Many hours' boiling with dilute acid are required to effect complete hydrolysis.

Abderhalden, Medigreceanu and Pincussohn have compared the hydrolysis by acids and alkalies. Alkali seems to produce the most complete hydrolysis. Abderhalden and Brahm found that a resistant body formed from silk could only be completely hydrolysed by alkali. The main difference in the results of hydrolysis by acids and alkalies consists in the products; the optically active amino acids are completely racemised by the alkali, whereas acids only produce partial racemisation. Arginine is destroyed by boiling with alkali and converted into ornithine and ammonia. Cystine is also decomposed by alkali.

The Isolation and Estimation of the Units.

The amino acids which compose the protein molecule can be divided into two main groups:—

A. The monoamino acids, including proline and oxyproline.

Tyrosine and cystine are usually treated independently.

B. The diamino acids, including histidine.

The three compounds in this group were formerly called the hexone bases on account of their basic properties and the fact that each of them contains six carbon atoms.

The remaining unit, tryptophane, is almost completely destroyed by hydrolysis by acids; it can only be isolated after hydrolysis by trypsin.

Ammonia is estimated by hydrolysing the protein with concentrated hydrochloric acid, removing the great excess of acid by evaporation under reduced pressure, adding excess of magnesia, distilling off the ammonia *in vacuo* and collecting it in excess of standard acid. This operation is usually carried out in the determination of the distribution of the total nitrogen amongst the two main groups. The

estimation of ammonia is frequently combined with the estimation of the diamino acids (p. 34).

The separation and estimation of the two main groups of amino acids is generally not carried out in one experiment, but only when the amount of protein available is small, as very different quantities of material are required. Thus, the diamino acids can be determined in 25-50 grammes of protein with considerable accuracy, whereas the monoamino acids can only be determined with fair accuracy when 250-500 grammes of protein can be used. The two processes, of which the details are given under the two sections, may be combined as follows:—

The protein is hydrolysed by boiling for fifteen to twenty-four hours with six times the quantity of 25-30 per cent. sulphuric acid. The solution is neutralised with baryta and the filtrate and washings from the barium sulphate are evaporated down to a small volume. Tyrosine (and cystine) crystallise out. The filtrate is diluted with water and sulphuric acid added till the content of acid is 5 per cent. The diamino acids are then precipitated with phosphotungstic acid (pp. 65, 75); from this precipitate they are obtained by decomposition with baryta and separated by means of their silver compounds as described in section B (p. 34). The filtrate is freed from phosphotungstic acid and sulphuric acid with baryta, excess of which is removed with carbon dioxide and sulphuric acid, and then treated for the other monoamino acids as described in section A (p. 17).

On the whole it is not advisable to combine the two processes, since the phosphotungstic acid precipitation does not effect a perfect separation of the two groups.

A. The Monoamino Acids.

The Isolation and Estimation of Tyrosine and Cystine.

Two of the amino acids, namely, tyrosine and cystine, are characterised by their extremely slight solubility in neutral aqueous solutions. They are therefore easily obtained after hydrolysis by acids by neutralising and concentrating the solution, when they crystallise out.

Hydrolysis by sulphuric acid possesses one great advantage over that by hydrochloric acid, as it can be subsequently completely and easily removed by baryta.

The protein is hydrolysed by boiling with five to six times its quantity of 25 per cent. sulphuric acid for twelve to fifteen hours; the solution, after filtration, is diluted with twice its volume of water and neutralised with barium carbonate, or a strong solution of baryta, the excess of which is then quantitatively removed by dilute sulphuric acid. The solution, together with the water used in thoroughly washing the precipitate of barium sulphate, is then evaporated down, until these acids crystallise out. They are filtered off, the filtrate is concentrated, and further crops of crystals are removed until the mother liquor no longer gives Millon's reaction for tyrosine. The amount of cystine in most proteins is so small that the product generally consists only of tyrosine. It is purified by recrystallisation from water, decolorisation of the solution being effected by charcoal. The yield of tyrosine so obtained is the measure of its amount in the protein.

On account of the insolubility of these compounds and the difficulty of filtering and completely washing the barium sulphate precipitate in order to abstract from it the whole of the tyrosine, Abderhalden and Teruuchi, in the case of silk, have hydrolysed the protein with hydrochloric acid, the greater part of which was then removed by evaporating several times *in vacuo* after diluting the concentrated solution with water; the remainder of the hydrochloric acid was then estimated in a small aliquot portion, and the main bulk neutralised with the calculated amount of caustic soda. The tyrosine then crystallised out, and was purified by recrystallisation from water.

When large quantities of protein are under investigation, the removal of the hydrochloric acid, after evaporation *in vacuo*, is effected by treating the solution with cuprous oxide until it is green in colour, filtering off and washing the cuprous chloride, and removing

dissolved copper by hydrogen sulphide. A current of air is then passed through the solution to remove the hydrogen sulphide, and the remainder of the hydrochloric acid is either neutralised with the calculated quantity of soda or is removed by treating with silver carbonate. The solution on concentration deposits the tyrosine.

Levene prefers the use of hydrochloric acid to that of sulphuric acid for separating tyrosine on account of the difficulty of completely extracting it from the barium sulphate precipitate and of obtaining it in a state of purity. His procedure is the following: The protein is hydrolysed with concentrated hydrochloric acid; the solution is concentrated and saturated with gaseous hydrochloric acid. Glutamic acid hydrochloride separates out. The filtrate and washings from this precipitate are concentrated *in vacuo* to remove the greater part of the hydrochloric acid. The solution is then diluted to 7 litres (for 400 grammes protein) and boiled with lead oxide till its reaction is alkaline. The lead oxide is prepared by precipitation with baryta, washed by decantation and preserved in the form of a paste. The precipitate of lead oxychloride is filtered off when the solution has cooled. It retains the resinous matters and a nearly colourless filtrate results. The remainder of the chlorine, which is estimated in an aliquot portion, is removed by means of the calculated quantity of silver sulphate, the excess of lead by adding sulphuric acid and passing in hydrogen sulphide, and of sulphuric acid by baryta. On concentrating the solution to one-seventh almost pure tyrosine separates out, which can be filtered off, washed, dried, and weighed. A portion of the other amino acids can be obtained by further concentration, and treated for leucine and valine. The diamino acids are then precipitated (p. 11) and the filtrate is treated for the other monoamino acids.

The estimation of tyrosine is thus effected by weighing the crystals which are obtained by the above procedures.

A new method of determining the presence of tyrosine by bromination was introduced by Horace Brown and employed by Adrian Brown and Millar in 1906 for estimating the rate at which tyrosine is split off from proteins by the action of trypsin. This method has recently been used for the estimation of tyrosine in proteins by Plimmer and Eaves.[1] The values for most proteins agree very closely with those obtained by isolating and weighing the tyrosine, but are slightly higher.

The separation of cystine and tyrosine when they are obtained

[1] As yet unpublished.

together was described by Mörner in 1901. The protein—hair, keratin from horn, egg-shells, etc.—was boiled with five times its quantity of 13 per cent. hydrochloric acid under a reflux condenser on a water-bath for six to seven days. The solution was then decolorised with charcoal and evaporated *in vacuo*, and the residue dissolved in 60-70 per cent. alcohol. The two acids then crystallised out on neutralising with soda, and were separated by fractional crystallisation from ammonia; if much tyrosine was present it separated out first, but if cystine exceeded tyrosine in quantity this compound crystallised out first; the remainder was only separated with difficulty.

Embden separated the mixture of the two acids by means of very dilute nitric acid, in which tyrosine is very easily soluble, but cystine with difficulty.

Their separation may also be effected by precipitation with mercuric sulphate in 5 per cent. sulphuric acid solution in which the mercury compound of tyrosine is soluble (Hopkins and Cole).

Winterstein, in 1901, showed that cystine was precipitated by phosphotungstic acid, but the observation seems to have been overlooked. Cystine and tyrosine are best separated by means of this reagent. The mixture is dissolved in 5 per cent. sulphuric acid and treated with excess of phosphotungstic acid solution. Cystine phosphotungstate generally separates out in a crystalline condition. From this precipitate the cystine can be obtained by the usual method (see under diamino acids), but a large excess of baryta must be avoided as cystine is readily decomposed by alkalies. Cystine crystallises out on neutralising the filtrate from the barium phosphotungstate. Winterstein decomposed the phosphotungstate with hydrochloric acid. The precipitate is made into a paste with water, placed in a separating funnel and treated with small quantities of concentrated hydrochloric acid. Ether is added and the mixture well shaken. At first an emulsion is formed, but on adding more ether and acid and thoroughly shaking a clear ethereal solution of phosphotungstic acid settles to the bottom; if this does not occur, decomposition is not complete and more acid must be added and the shaking repeated. The middle layer containing the cystine hydrochloride is separated; the other layers are treated once more in the same way and the middle layers are combined. Ether is removed from the acid solution by warming on the water-bath and the cystine is separated by exactly neutralising with soda.

Folin prepares cystine from wool by boiling it with concentrated hydrochloric acid in the proportion of 100 grammes wool to 200 c.c.

acid for three to five hours. The hot solution is then neutralised to congo red by adding sodium acetate in the form of crystals. Almost the whole of the cystine separates out on standing. After several hours the precipitate is dissolved in 3-5 per cent. hydrochloric acid, the solution is boiled with charcoal, and the hot colourless solution is neutralised as before by adding a hot concentrated solution of sodium acetate. Cystine separates out in the characteristic hexagonal plates as the solution cools. No data of the yield are given.

The mother liquor on dilution and on standing deposits tyrosine. This is most readily purified by dissolving in hydrochloric acid, decolorising the solution with charcoal, and then neutralising exactly with ammonia, when almost pure tyrosine separates out.

Some experiments which have been carried out in my laboratory with hair and other keratins by this process have sometimes given a mixture of cystine and tyrosine on neutralising with sodium acetate; sometimes tyrosine has separated out first. This agrees with Mörner's results in which the first product to separate out was the one present in greatest amount. The separation was effected by phosphotungstic acid.

The Isolation and Estimation of Tryptophane.

Tryptophane is not obtained in any large amount by the hydrolysis of proteins by acids and is best prepared by the action of trypsin. According to Hopkins and Cole, the protein is digested in alkaline solution by trypsin, until the solution gives a maximal coloration when tested with bromine water; the solution is then acidified, boiled, and filtered. The clear solution (better after concentrating *in vacuo* and filtering off tyrosine, which crystallises out) is acidified with sulphuric acid until it contains 5 per cent., and then mercuric sulphate dissolved in 5 per cent. sulphuric acid is added as long as a precipitate, which contains tryptophane, cystine and tyrosine, is formed. The precipitate is freed from tyrosine by washing with 5 per cent. sulphuric acid in which the tyrosine compound is soluble, that is, until the washings no longer react with Millon's reagent. It is then decomposed by sulphuretted hydrogen, and the solution containing cystine and tryptophane is again acidified with sulphuric acid to 5 per cent. and fractionally precipitated with the mercuric sulphate reagent. The cystine is thrown down first, and filtered off, and then the tryptophane is precipitated. The precipitate is again decomposed by hydrogen sulphide, and the solution, freed from sulphuric acid, is evaporated down, alcohol being continually added to hasten the evaporation and prevent decomposition of the tryptophane, which is estimated by weighing.

Neuberg and Popowsky, as also Abderhalden and Kempe, have introduced a few alterations in the procedure, such as evaporation *in vacuo*, and Levene and Rouiller suggested in 1907 that the tryptophane, on account of its proneness to decompose on evaporation of its solution with consequent loss, be estimated colorimetrically; the mercury sulphate precipitate is decomposed, and the solution, freed from hydrogen sulphide, is titrated with bromine water in presence of amyl alcohol. Both cystine and tyrosine react with bromine water; the latter can, however, be removed, but for the former a correction has to be made. Up to the present no values concerning the amount of tryptophane in various proteins by this method have appeared, and it will be of interest to see if the values so obtained are very much higher than those obtained by crystallisation of the tryptophane.

The Isolation and Estimation of the other Monoamino Acids.

For the preparation and estimation of the monoamino acids, hydrolysis by hydrochloric acid is more convenient than that by sulphuric acid. It was formerly carried out in the presence of stannous chloride (Hlasiwetz and Habermann) in order that the solution should remain colourless, instead of becoming dark brown, but this addition is not essential, as was shown by Cohn, and was not used by E. Fischer in his researches. Hydrolysis by hydrochloric acid is carried out by heating the protein with three times its quantity of concentrated hydrochloric acid of specific gravity 1·19, when it gradually passes into solution, if the flask in which it is contained be occasionally shaken and slightly warmed, and then boiling under a reflux condenser for ten to twenty-four hours or longer, depending on the particular protein, and until the biuret reaction has completely disappeared. The solution, which may become at first violet, becomes finally of a dark-brown colour, and a portion of the hydrochloric acid is evolved as gas; it is filtered from humin substances (secondary products probably arising from tryptophane and carbohydrate) and fatty material through a Buchner funnel covered with linen, and well washed with water.

(1) Isolation of Glutamic Acid as Hydrochloride.

The solution of amino acids in 25 per cent. hydrochloric acid is concentrated *in vacuo* to a small volume, and glutamic acid, if present in any large amount, is removed as its hydrochloride by saturating the solution with dry gaseous hydrochloric acid and allowing to stand at 0° C. for some days, when glutamic acid hydrochloride crystallises out. This occurs in the case of caseinogen and certain vegetable proteins, which contain from 10-40 per cent. of this amino acid. The glutamic acid hydrochloride is filtered off after adding an equal volume of ice-cold alcohol, redissolved in water and boiled with baryta to remove ammonia. The barium is removed with sulphuric acid and the glutamic acid is again precipitated as hydrochloride by saturating the solution with gaseous hydrogen chloride. The mother liquor on further concentration may give further crops of glutamic acid hydrochloride. These are treated in the same way as the first crop.

(2) Esterification and Isolation of Glycine as Ester Hydrochloride.

The filtrate from the glutamic acid hydrochloride, to which the mother liquors from the recrystallisations are added, is again concentrated *in vacuo* at a low temperature to a thick syrup; this is dissolved in absolute alcohol (3 litres to 1 kilo protein), and the amino acids are esterified by saturating the alcohol with dry gaseous hydrochloric acid at the ordinary temperature and then warming on the water-bath for half an hour. In the process of esterification a large amount of water is formed, which prevents its completion; the alcohol is therefore evaporated off *in vacuo* at a temperature below 50° C., and the resulting syrup again dissolved in absolute alcohol and saturated with dry gaseous hydrochloric acid. In some cases it may be necessary to repeat this operation once more.

The esterification, according to Osborne and Jones, is more advantageously effected by the method of Phelps and Tillotson. The concentrated solution of amino acid hydrochlorides is dissolved in alcoholic hydrochloric acid and zinc chloride is added as a catalyst. The solution is maintained at a temperature of 100° C. and the vapours of absolute alcohol containing some hydrochloric acid are passed into the solution. The water arising during the process is removed by the alcohol vapours as fast as it is formed, and complete esterification results in a shorter time.

At this stage, glycine, if it occurs in the protein, *e.g.*, in gelatin, in any considerable amount, is separated as glycine ester hydrochloride by concentrating *in vacuo* at 40° C. to two-thirds and seeding the solution with a crystal of this compound and allowing to stand for twenty-four hours at 0° C. The precipitate is filtered off while the liquid is kept cold and is washed with ice-cold alcohol; the mother liquor, on further concentration *in vacuo* and saturation again with hydrochloric acid, may give another crop of glycine ester hydrochloride, which is treated in same way. The glycine ester hydrochloride is dried *in vacuo* over lime and sulphuric acid, and is purified by recrystallisation from absolute alcohol, charcoal being used to decolorise the solution. Almost the whole of the glycine may be isolated in this way.

The filtrate containing the esters of the hydrochlorides of the other amino acids and the filtrate from the recrystallised glycine ester hydrochloride are combined and concentrated to a syrup *in vacuo* at 40° C.; the process of esterification is best repeated again, and any further quantities of glycine ester hydrochloride separated.

(3) Extraction of the Esters of the Amino Acids.

The solution is concentrated *in vacuo* at 40° C. to a syrup. The esters are then liberated from their hydrochlorides by one of the following methods:—

(*a*) About one-third to one-half the volume of water is added to dissolve the syrup, and, if 1 kilo of protein has been used, the solution is divided into two or four portions for convenience and to ensure the subsequent thorough cooling; to each portion two or three volumes of ether are added, and the mixture is thoroughly cooled in a freezing mixture of ice and salt; strong caustic soda (33 per cent.) is now added till the free hydrochloric acid is neutralised; this can be tested by adding a small quantity of a saturated solution of potassium carbonate. The feebly basic esters of aspartic and glutamic acids, which are very sensitive to free alkali, are thus liberated and are dissolved by the ether, which is quickly poured off and replenished by a fresh quantity. Caustic soda and solid potassium carbonate added in small portions at a time set free the other esters from their hydrochlorides; these are dissolved by the ether, which is frequently renewed throughout the process and thoroughly mixed with the mass of salt and potassium carbonate; sufficient caustic soda must be added to combine with the whole of the hydrochloric acid, and as much potassium carbonate as is necessary to form finally a pasty mass in order that the esters, which are very easily soluble in water, are salted out and dissolved by the ether. A large amount of ether is required for this extraction, which is continued until the ether separates in a colourless state, and an essential condition is that, throughout the process of extraction the various portions be kept thoroughly cold by shaking in the freezing mixture.

The several ethereal extracts are each dried by shaking for about five minutes with potassium carbonate; they are then combined together and allowed to stand for twelve hours with anhydrous sodium sulphate.

The ether is next evaporated off, preferably in small quantities at a time and *in vacuo* at the ordinary temperature; in this way the lower boiling esters do not distil with the ether and the danger of decomposing them by a higher temperature is avoided. A brown oil, consisting of the esters of the amino acids, results; this is fractionally distilled *in vacuo*.

A considerable amount of the lower boiling esters nevertheless distils with the ether, especially those of alanine and glycine. Leucine ester has also been found in the distillate. They are recovered by shaking out the ether with dilute hydrochloric acid and evaporating the acid aqueous solution to dryness, when they remain as amino acid hydrochlorides. The glycine is separated as ester hydrochloride and the alanine, and leucine if present, are separated as esters by fractional distillation *in vacuo* (see p. 22). They may also be obtained by evaporating their solution with water, removing the hydrochloric acid with lead oxide and silver carbonate, the excess of these by hydrogen sulphide and fractional crystallisation. Leucine, if present, separates out first, and subsequently the alanine. A mixture of small quantities of glycine and alanine generally remains which is too small for further separation; it may be treated with picric acid (see p. 30).

By this method of extracting the esters from their hydrochlorides, neither that of tyrosine, which remains behind combined with alkali, nor those of the diamino acids, which are soluble with difficulty in ether, are obtained. This is advantageous for the subsequent process of separation, but the method has the disadvantage that the whole quantity of esters is not taken up by the ether. Extraction with chloroform after ether increases the amount of ester; it separates the pasty mass of potassium carbonate into particles which can be washed with the solvent; this extract contains the ester of tyrosine.

The main loss seems to be caused by the destruction of the esters by the alkali. In order to avoid this loss, the mass of carbonate is treated with excess of hydrochloric acid and evaporated down, the potassium chloride being filtered off as it separates out; the residue is extracted with alcohol and the above process of esterification is repeated. It is simpler to suspend the mass of carbonate in absolute alcohol and to saturate the solution with gaseous hydrochloric acid. The salts remain insoluble and are filtered off and the alcoholic solution is treated as has been described.

(*b*) In order to separate the amino acids as completely as possible, Fischer introduced another method of liberating the esters from their hydrochlorides, *i.e.*, treatment with sodium ethylate. The hydrochloric acid is removed as completely as possible by evaporation *in vacuo* and the mixture of ester hydrochlorides is dissolved in five times its quantity of absolute alcohol. The amount of chlorine is estimated in a small portion of this, and to the remainder the calculated quantity of sodium dissolved in absolute alcohol and freshly pre-

pared, so as to make a 3 per cent. solution, is added. The sodium chloride formed is filtered off. Its separation is greatly facilitated by the addition of ether and cooling to O° C. The alcohol is removed by evaporation *in vacuo*. A small quantity of the lower boiling esters of the amino acids passes into the distillate with the alcohol, but is recovered by acidifying with hydrochloric acid and evaporating when the amino acid hydrochlorides are obtained. A dark-brown oil again results, which is fractionally distilled *in vacuo*.

Although this method prevents loss by the action of alkali, the yield of the higher boiling fractions is not so great on account of the more complex nature of the mixture of esters. The residue which does not distil contains the tyrosine, the diamino acids and other substances.

(*c*) Instead of employing caustic soda and potassium carbonate for the liberation and salting out of the esters, Levene uses barium oxide for which he claims the following advantages :—

(i) On account of its small solubility in water, a large excess of alkali which causes saponification of the esters is avoided.

(ii) In neutralising the free acid the rise in temperature is less than with caustic soda.

(iii) When the second esterification is required it is more easily removed.

His procedure is as follows :—

The concentrated solution of the ester hydrochlorides is poured into a porcelain or enamelled vessel of capacity of 1 litre for every 125 to 150 grammes protein, and the flask is washed out with ice-cold baryta solution. The vessel is placed in a freezing mixture. When the contents are cold excess of crystallised baryta is added, and the mixture is thoroughly stirred with a wooden or porcelain spatula. The peculiar sticky mass in a few minutes becomes liquid and the solution becomes alkaline in reaction. Several volumes of ether are then poured over the solution. The ether is then poured off and a fresh quantity added. More baryta is added and the stirring continued. The ether is replenished and more baryta added several times. During the process the mixture becomes cloudy and pasty, but finally a light dry mass of baryta is left.

The ethereal solutions are treated as described in the (*a*) method.

For the second esterification process the residue is stirred up several times with water, filtered through asbestos and washed with water till no more organic matter is extracted. Most of the baryta remains

undissolved. The solution is freed from that which has dissolved by the equivalent quantity of sulphuric acid, acidified with hydrochloric acid and esterified in the usual manner.

(*d*) Pribram finds that the esters may be liberated very conveniently by the action of ammonia. His preliminary experiment with glycine gave a yield of 69 per cent. and an equally good yield of esters was obtained from gelatin. Abderhalden also finds this method very serviceable.

The concentrated solution of ester hydrochlorides is mixed with ether and surrounded with a freezing mixture. Ammonia, dried by passing through three towers of caustic soda and lime, is then passed into the solution. The ammonium chloride which is formed is filtered off and washed with absolute alcohol. The ether is distilled off and the esters fractionally distilled *in vacuo*.

(*e*) Zelinsky, Annenkoff and Kulikoff have quite recently suggested a still simpler procedure for obtaining the esters. The concentrated solution of the ester hydrochlorides is mixed with lead oxide (200 grammes to 100 grammes protein) and the mass is directly distilled *in vacuo*.

(4) Fractional Distillation of the Esters *in Vacuo*

The fractional distillation of the brown oil, which is obtained by either of these methods, is carried out firstly at a pressure of 10-12 mm. produced by a water pump, and then at a pressure of 0·5 mm. produced by a Geryck vacuum pump, as described by Fischer and Harries. In order to preserve the high vacuum in this process liquid air is used for condensing the alcohol vapour arising from the decomposition of the esters; carbonic acid has been used by other investigators, and Levene and Van Slyke have employed sulphuric acid, cooled by a freezing mixture, as an absorbent for this purpose. A small quantity of ester also passes over with the alcohol; this cannot be recovered if Levene and Van Slyke's modification be used. The accompanying figure (p. 23) shows the arrangement for the distillation at low pressure. At 10-12 mm. pressure the receiver should be cooled in a freezing mixture.

The temperatures at which the various fractions are collected are those of the vapours of the esters at 10 mm. pressure, and those of the water-bath at 100° C. and of an oil-bath, which replaces the water-bath for the higher temperatures up to 160° C., at 0·5 mm. pressure.

Formerly the lower boiling fractions were again distilled *in vacuo* so as to obtain a further fractionation, but each fraction, even then, did not generally contain a single ester of an amino acid. A second fractionation is therefore no longer carried out.

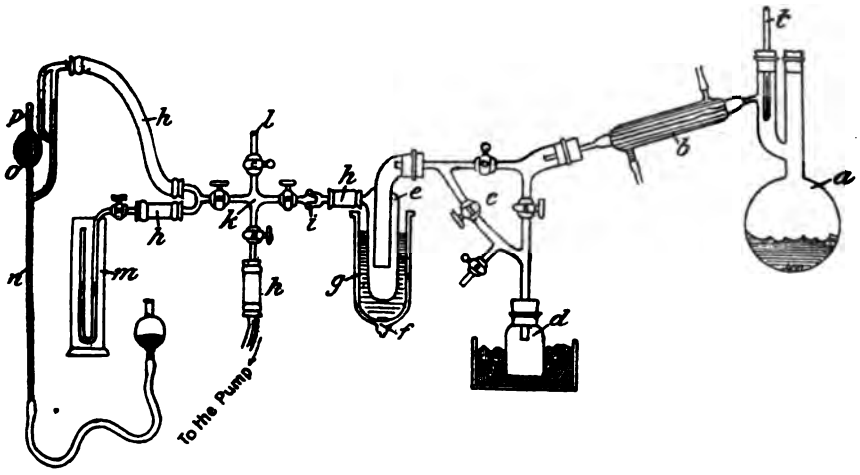


FIG. 1.

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In the case of the higher boiling fractions a second fractionation is not necessary, since the esters contained in them can be separated by their varying solubility in water, ether and petroleum ether. According to Osborne and Jones the distillation of the esters boiling above 110° C. can be dispensed with. An equally good separation of this residual mixture can be effected if it be carried out in the same way as is described for the esters after they have been distilled.

The following table shows the fractions which are collected, and the amino acid esters which they may contain:—

Temperature.	Pressure.	Esters of
Fraction I. To 60° (vapour)	10 mm.	Glycine, alanine, leucine, proline.
,, II. 60-90° (vapour), 100° (water-bath) .	10 mm.	Valine, leucine, proline.
,, III. 100° (water-bath)	0·5 mm.	Leucine, proline.
,, IV. 130-180° (oil-bath) . . .	0·5 mm.	Phenylalanine, glutamic acid, aspartic acid, serine.

(5) The Separation and Characterisation of the Individual Monoamino Acids.

The separation and characterisation of each constituent contained in an ester fraction has now to be carried out. A somewhat special process has to be adopted for each individual product.

(*a*) *Fractions I., II., III.*

The esters contained in these fractions are immediately reconverted into the amino acids. This is effected by boiling the fractions with 5-10 volumes of water under a reflux condenser for six to seven hours, until the alkaline reaction has disappeared.

If leucine be present in considerable amount, as may be the case in fraction III., the solution on cooling may deposit crystals of this substance. These are filtered off, washed with water, dried and weighed. Identification of the product is carried out as described below.

The three solutions are then evaporated to dryness *in vacuo* in weighed flasks and the amount of residue ascertained for each fraction.

Levene has found that leucine ester, like phenylalanine ester, is readily dissolved by ether from water. The ester fractions may therefore, before hydrolysis, be mixed with 3 volumes of water and extracted with ether, and the ether extract washed three times with water. The aqueous solution is then saponified by boiling and the extract, after removal of the ether, is treated in the same way.

Isolation and Characterisation of Proline.

Proline is the only product contained in these fractions which is soluble in alcohol; it is also much more easily soluble in water than the other products.

The dry residues are therefore extracted several times with boiling absolute alcohol; these extracts on cooling frequently become turbid and on standing deposit other amino acids which, though insoluble in alcohol, are dissolved when proline is present. They are filtered off and returned to the portion insoluble in alcohol.

The combined alcoholic extracts of the three fractions are again evaporated to dryness *in vacuo* and the residue is treated several times with cold absolute alcohol. A considerable amount is undissolved; as before this is returned to the insoluble portion.

The alcoholic solution is again evaporated to dryness *in vacuo* and

extracted with cold absolute alcohol, and this operation is continued until all the insoluble amino acids are removed.

The final alcoholic solution is evaporated to dryness and the residue weighed. As thus obtained, the proline is a mixture of the optically active and the racemic forms. These are separated by conversion into their copper salts by boiling with freshly precipitated copper oxide. The resulting dark-blue solution is evaporated to dryness, and the residue is treated with absolute alcohol which dissolves the copper salt of the optically active proline. This solution on concentration yields the greater part of the compound in a crystalline state, but the remainder is amorphous. The copper salt of the racemic proline, which is insoluble in alcohol, is purified by crystallisation from water.

The identity of the compounds is established by a determination of the water of crystallisation and of the copper. The racemic copper salt contains 2 molecules of water of crystallisation, and in this state it is dark blue in colour; in the anhydrous state its colour is violet. Further characterisation is obtained by preparing proline from it. The copper salt is dissolved in water and decomposed with hydrogen sulphide. The filtrate is concentrated to a small volume and precipitated with alcohol. The product, crystallised from alcohol, is obtained in flat needles. *l*-proline has a sweet taste, melts at 206-209° C. and has a rotation of $[\alpha]_{20}^{D} = -77{\cdot}4°$.

The amount of proline in the protein is given by the yield of the two copper salts obtained in a pure state. The actual proline content of the protein is considerably larger than the figures given in the tables (pp. 53-62).

The real proline content can be accurately determined, as Van Slyke has shown by his nitrous acid method (p. 69), by a determination of the total and amino-nitrogen of the product soluble in absolute alcohol. The alcoholic solution is made up to a definite volume and aliquot portions are taken for these estimations. The difference gives the amount of nitrogen present as proline from which the amount of proline can be calculated. In the case of caseinogen the proline content was found to be 6·7 per cent., a figure which is twice that found by Abderhalden. It agrees with that found by Engeland by his method of methylation (p. 43).

Isolation of Glycine, Alanine, Valine, Leucine, and Isoleucine.

These five amino acids are present together in varying proportions in the residues which are insoluble in absolute alcohol. Their separation is only effected with great difficulty, and the procedure depends very largely upon which amino acids are present in the several fractions.

In each case the residue is dissolved in water and, if necessary, the solution is decolourised by boiling with charcoal. The aqueous solutions are concentrated and fractionally crystallised; the final mother liquor is evaporated to dryness. Each fraction is dried and weighed. Indications of the constituents of each fraction may be obtained:—

1. By elementary analysis of the carbon, hydrogen, and nitrogen content.

2. By determining the melting-point; the substance must be rapidly heated. Glycine melts at 240°, alanine about 297°, leucine about 300°, and valine about 315°.

3. By the taste. Glycine and alanine have a sweet taste; valine is less sweet and it leaves a bitter after-taste; leucine is insipid and slightly bitter.

The residues from fraction I. will contain chiefly glycine and alanine; from fraction II. valine, leucine and isoleucine; from fraction III. leucine and isoleucine.

Separation of Valine from Leucine and Isoleucine.

These three compounds are the most difficult to separate from each other. Their separation has really only been accomplished by chance.

In those cases where the isolation of the individual substance has succeeded it has been effected by the fractional crystallisation of the amino acids themselves and of their copper salts and by the different solubility of the compounds in methyl alcohol. Valine is soluble in methyl alcohol, isoleucine is insoluble in the cold solvent, but soluble when hot. On cooling the solution, however, the presence of valine prevents its separation. The copper salt of leucine is very insoluble, but the mixed copper salts are relatively soluble. Leucine and isoleucine were first separated by F. Ehrlich by the different solubility of their copper salts in methyl alcohol; that of leucine is insoluble. The separation is most tedious and not at all satisfactory.

In order to give us more information about the constituents of this

fraction, Levene and Van Slyke have worked out a method which depends upon the precipitation of leucine and isoleucine as their lead salts from ammoniacal solution and the subsequent separation of these two amino acids by means of the different solubility of their copper salts in methyl alcohol. The details were published in 1909 and are as follows:—

The mixture is carefully analysed and the amount of leucine + isoleucine is calculated from the carbon content:—

$$\left.\begin{array}{l}\text{Leucine and isoleucine contain } 54{\cdot}92 \text{ per cent. C.}\\ \text{Valine contains } \quad . \quad . \quad . \; 51{\cdot}24 \quad \text{,,} \quad \text{,,}\end{array}\right\} \text{difference} = 3{\cdot}68 \text{ per cent.}$$

$$\frac{\text{Per cent. carbon} - 51{\cdot}24}{3{\cdot}68} = \text{per cent. of leucine isomers in the mixture.}$$

The mixture is pulverised and suspended in 7 parts of water and the water is raised to the boiling-point: for each gramme of substance 1·5 c.c. of concentrated ammonia solution is then added. The flask is stoppered and shaken so as to dissolve the amino acids; if necessary the solution may be again heated.

Four c.c. of 1·1 M lead acetate solution (sp. gr. 1·254 at 20°) for each gramme of leucine and isoleucine are slowly added to the hot solution, which is thoroughly stirred during the addition. The solution is then cooled in ice-water and after one to two hours is filtered through a Buchner funnel, or a Gooch crucible, according to the amount of precipitate. The solid matter is pressed down so as to remove the mother liquor as completely as possible, and it is washed firstly with 90 per cent. alcohol, then with ether, and dried *in vacuo* over sulphuric acid.

It is curious that the presence of valine facilitates the separation of the lead salts of the leucine isomers.

If the proportion of isomers: valine be less than 2 : 1 the precipitation is not so complete. In these cases less lead acetate solution (3·7 c.c.) should be taken and the filtrate concentrated *in vacuo* till the percentage of valine reaches 10. Ammonia is again added and the precipitate treated as before. It is preferable to treat the filtrate once more in the above manner after the valine has been separated.

Isolation and Characterisation of Valine.

The filtrate is freed from lead by means of hydrogen sulphide, and the solution, filtered from lead sulphide, is evaporated to dryness. The dry residue is treated with an alcohol-ether mixture (3 : 1) to extract acetic acid and ammonium acetate. The small amount of valine which dissolves is recovered by again evaporating to dryness and extracting with alcohol and ether.

Pure valine generally remains; it is identified by recrystallisation from water, elementary analysis, and rotation in 20 per cent. hydrochloric acid $[\alpha]_{20}^{D} = +28{\cdot}8°$; a rotation of 26°-28° is generally found as racemisation occurs in the process.

If analysis shows leucine to be present the above treatment must be repeated.

Treatment of the Lead Salts of Leucine and Isoleucine.

The purity of the lead salt, as obtained above, is tested by analysis. This is performed by dissolving about 0·3 gm. in 5 c.c. of normal nitric acid in a 100 c.c. beaker and precipitating the lead with 5 c.c. of normal sulphuric acid followed by 50 c.c. of absolute alcohol. The lead sulphate, which settles rapidly in a granular form, is collected after about fifteen minutes in a Gooch crucible and washed with 95 per cent. alcohol acidified with sulphuric acid. The crucible is placed in another crucible, heated gently until the alcohol is driven off and then with the full heat of the Bunsen burner for ten minutes.

If the lead content be too high, due to contamination with the lead salt of valine, the mixture is purified by dissolving it, after thorough pulverisation, in 5 parts of hot water + ¼ part of glacial acetic acid and reprecipitating by adding 0·5 c.c. of concentrated ammonia solution for every gramme of salt. The precipitate is collected and treated as above under the conditions where the amount of valine present is small.

The last portions of the mixed leucines are recovered by repeating the entire process.

Separation of Leucine and Isoleucine.

The mixed lead salts are dissolved in 15-20 parts of hot water + ¼ part of glacial acetic acid, and hydrogen sulphide is passed into the solution. The filtrate from the lead sulphide is evaporated *in vacuo* to dryness, and the residue is washed with a mixture of equal parts of alcohol and ether to remove acetic acid.

Since these isomers have been shown not to be racemised by heating with acids, the composition of the mixture can be ascertained by determining the rotation in 20 per cent. hydrochloric acid:—

$$\left.\begin{array}{l} d\text{-isoleucine has } [\alpha]_{20}^{D} = +37{\cdot}4^{\circ} \\ l\text{-leucine} \quad " \quad [\alpha]_{20}^{D} = +15{\cdot}6^{\circ} \end{array}\right\} \text{difference} = 21{\cdot}8^{\circ}$$

$$\text{hence percentage of } d\text{-isoleucine} = 100 \times \frac{\alpha - 15{\cdot}6}{21{\cdot}8}$$

$$" \quad " \; l\text{-leucine} = 100 \times \frac{37{\cdot}4 - \alpha}{21{\cdot}8}$$

From the weight of the mixed amino acids and these data the amount of each isomer can be calculated.

The mixed isomers are converted into their copper salts by boiling with excess of copper oxide, which is thoroughly boiled out with water to remove the last traces of the copper salt of leucine which is pale blue in colour and very insoluble.

The solution of the copper salts is evaporated to dryness *in vacuo*. The dry and finely pulverised copper salts are then shaken in a shaking machine with 94 per cent. methyl alcohol. The insoluble copper salt of leucine is filtered off and washed with solvent. The soluble copper salt of isoleucine may be contaminated with some of the copper salt of leucine. It is therefore decomposed with hydrogen sulphide, reconverted into copper salt and again extracted with methyl alcohol. Both the leucine and isoleucine are obtained in the usual way from the copper salt and recrystallised from water.

They are identified by elementary analysis, rotation, and estimation of copper in their copper salts.

Separation of Glycine and Alanine.

By the fractional crystallisation of the amino acids obtained from the esters the following fractions may be obtained:—

1. Valine + leucines. These are treated as above described.

2. Valine + alanine. These are separated by fractional crystallisation and also by the fractional crystallisation of the copper salts.

3. Alanine. This is purified by recrystallisation from dilute alcohol.

4. Alanine + glycine. There are two methods of separating these compounds :—

(*a*) By reconverting the mixture into esters, separating the glycine as ester hydrochloride and distilling the alanine ester, which is then decomposed by boiling with water and the alanine obtained by crystallisation.

(*b*) By precipitating the glycine as picrate (Levene). The mixture is dissolved in a small quantity of hot water and an equal quantity of picric acid, dissolved in alcohol, is added. Glycine picrate crystallises out on cooling. The pure compound melts at 190°. The alanine may be obtained from the filtrate by acidifying, extracting the excess of picric acid with ether, removing the acid and then crystallising out.

The glycine is identified by the melting-point and analysis of its ester hydrochloride and picrate; its amount in the protein is given by the yield.

Alanine is identified by elementary analysis and its rotation in hydrochloric acid solution. The amount in the protein is also given by the yield.

(*b*) *Fraction IV.*

(*a*) Phenylalanine.

The ester of phenylalanine differs from the esters of aspartic acid, glutamic acid and serine, which are present with it in this fraction, by being only slightly soluble in water.

The mixed esters are dissolved in 5 volumes of water. If a large amount of phenylalanine be present, it may separate in the form of oily drops. The aqueous solution is extracted with an equal volume of ether. The ether extract is then washed several times with water to remove the last traces of any of the other esters which may have been dissolved by the ether. The ether is removed by distillation and the ester is hydrolysed by evaporation with concentrated hydrochloric acid. The resulting phenylalanine hydrochloride is purified by crystallisation from hydrochloric acid, and can be identified by an estimation of its content in chlorine.

The free amino acid is obtained from the hydrochloride by treating with sodium acetate, or ammonia, and precipitating from hot aqueous solution with alcohol. A determination of the melting-point of 283° C and rotation of $[\alpha]_{20}^{D} = -35{\cdot}1$ in aqueous solution characterises the compound

(*b*) Aspartic Acid.

The esters in the aqueous solution from which the phenylalanine ester has been extracted with ether are saponified by boiling with baryta; the solution of baryta is prepared by dissolving twice the quantity of barium hydrate to that of ester in sufficient hot water, filtering, and allowing to cool. The clear solution is poured off from the crystals and to it is added the solution of esters. Hydrolysis is then effected by heating for two hours on the water-bath. The solution is allowed to stand for several days to allow the barium salt of racemic aspartic acid to crystallise out.

The barium aspartate is decomposed with sulphuric acid, the barium sulphate filtered off, and the excess of sulphuric acid quantitatively removed with baryta. Pure aspartic acid crystallises out from the solution on evaporation. It is identified by analysis and by the analysis of its copper salt.

The remainder of the aspartic acid is isolated after removal of glutamic acid as hydrochloride (*c*).

The solution is evaporated *in vacuo* to remove as much hydrochloric acid as possible. The residue is dissolved in water and boiled with yellow lead oxide until a test portion of the cold solution no longer gives a reaction for chlorine. The filtered solution is freed from lead by hydrogen sulphide and the filtrate from lead sulphide is evaporated to a small volume, when aspartic acid crystallises out. The mother liquor contains principally serine, but more aspartic acid and other products are also present.

Note.—Osborne and Liddle have observed that an intermediate fraction between fractions III. and IV. containing aspartic ester and leucine ester and possibly also phenylalanine ester may distil. The separation of leucine and aspartic acid (or glutamic acid) is impossible by fractional crystallisation. The fraction should be treated as described and the leucine separated from the aspartic acid by neutralising with soda and crystallising. Leucine results. On acidifying the filtrate and again crystallising the aspartic acid is obtained.

(*c*) Glutamic Acid.

The filtrate from the barium aspartate is exactly freed from barium by sulphuric acid and the solution is evaporated to dryness *in vacuo*. The residue is dissolved in water, the solution decolourised, if necessary, by boiling with charcoal and the glutamic acid is precipitated as hydrochloride by passing in dry gaseous hydrogen chloride. A further quantity of glutamic acid hydrochloride may be obtained from the mother liquor by concentration and similar treatment. Practically the whole of the glutamic acid present in the protein is thus obtained as hydrochloride. The larger portion is separated directly, before the mixture of amino acids is esterified.

Glutamic acid is obtained from the hydrochloride by treatment with the calculated quantity of caustic soda to combine with the hydrochloric acid and by crystallisation from water, in which it is soluble, when pure, with some difficulty. Elementary analysis of the free acid, or of its hydrochloride, determines its identity and its weight gives the amount in the protein.

(*d*) Serine.

It is most difficult to isolate serine and obtain it in a pure state. The solution from which the active aspartic acid has crystallised out is neutralised, if acid, with caustic soda and concentrated. Serine crystallises out in crusts of monoclinic crystals, and is identified by its melting-point of 240° and elementary analysis.

Its β-naphthalene sulphonyl-derivative,

$$C_{10}H_7SO_2Cl + H_2N.CH\begin{matrix}CH_2OH\\COOH\end{matrix} = HCl + C_{10}H_7SO_2.NH.CH\begin{matrix}CH_2OH\\COOH,\end{matrix}$$

which is prepared by shaking in alkaline solution with β-naphthalene sulphonyl-chloride, serves for the isolation of the remainder from the filtrate. This compound is very suitable for its characterisation. (M.P. = 214° C. corr.)

The Distillation Residue.

A dark reddish-brown and thick syrupy residue remains in the distilling flask and sometimes crystals are observed clinging to the walls of the vessel. The mass consists chiefly of the anhydrides of the amino acids. Leucinimide may be extracted by boiling it with ethyl acetate. The residue is generally so small in amount that it is not further examined, but if this be necessary the mass is boiled with baryta for sixteen hours, the baryta removed and the process of separation repeated.

The Isolation of Oxyproline.

Only in a few cases has this compound been isolated from the products of hydrolysis of proteins, since its separation is extremely laborious. Its ester is not extracted by ether, and it consequently remains behind in the mass of carbonate; if its isolation be required a second esterification is generally not performed.

The mass of carbonate is treated as previously described (pp. 20, 21). The aqueous solution is evaporated *in vacuo* to remove hydrochloric acid as completely as possible. The organic matter is dissolved in water so that its content is about 1 per cent.; sulphuric acid is added and the diamino acids are precipitated with phosphotungstic acid (see p. 11). The excess of reagents are removed from the filtrate with baryta, the solution is concentrated *in vacuo* to a small volume and hydrochloric acid is removed with silver sulphate. The silver and sulphuric acid are then precipitated in the usual way and the solution is evaporated *in vacuo* to a small volume and then allowed to stand in a desiccator over sulphuric acid. Oxyproline slowly crystallises out.

Oxyproline is more easy to obtain when the esters have been separated with sodium ethylate. It is then present in the distillation residue; this is treated in a similar way. It is identified by its melting-point of 270°, rotation of $[\alpha]_{20}^{D} = -81{\cdot}04°$ in aqueous solution and by conversion into its β-naphthalene sulphonyl-derivative.

B. The Diamino Acids.

The isolation and estimation of the three compounds—arginine, histidine, lysine—is carried out by the method described by Kossel and Kutscher in 1900, which was slightly modified in 1903 by Kossel and Patten. Further modifications and improvements have been added by Steudel, Weiss, and Osborne and his associates Leavenworth and Brautlecht. The method is based upon the earlier work of Drechsel, Hedin, and Kossel, and depends upon the precipitation of arginine and histidine as their silver salts, their separation by difference in solubility in water and in strongly alkaline solution, and the precipitation of lysine from the filtrate by phosphotungstic acid, and then by picric acid.

It is carried out as follows:—

I. Hydrolysis and Estimation of Protein.

About 25-50 grammes of protein are hydrolysed by boiling with a mixture of three times the weight of concentrated sulphuric acid and six times the weight of water under a reflux condenser firstly for one to one and a half hours on a water-bath until frothing has ceased and then in an oil-bath at 105° for fourteen, or better twenty-four, hours. The exact amount of protein is then estimated by making the volume up to 1 litre with water, and determining the nitrogen in 5 or 10 c.c. by Kjeldahl's method; from this figure the amount of protein can be calculated, if the amount of nitrogen in it be known.

II. Removal of Sulphuric Acid. Estimation of Ammonia and Humin Nitrogen.

The acid solution is heated to boiling and treated with a hot concentrated solution of baryta until the reaction is only faintly acid and almost the whole of the sulphuric acid is precipitated as barium sulphate, which is filtered off by suction and thoroughly washed with boiling water, by stirring up and raising to the boiling-point. This should be repeated twice or until the filtrate gives no precipitate

with phosphotungstic acid. The filtrate and washings are evaporated down best *in vacuo* at 70° C. and again made up to 1 litre. A determination of the nitrogen in 5 or 10 c.c. of this solution gives by difference the amount of nitrogen contained in the melanin, which is carried down by the barium sulphate. It is known as "humin nitrogen I.".

In this liquid two determinations are made of the amount of nitrogen present as ammonia, by distilling portions of 100 c.c. with magnesium oxide.

The ammonia is removed from the remainder by evaporating with magnesia, or better barium carbonate, on the water-bath.

The two portions, freed from ammonia, are then combined, and made alkaline with baryta, or barium carbonate.

The separate solutions are now combined, the precipitate of barium carbonate and barium sulphate is filtered off and washed by boiling with water three times; the excess of barium is removed from the filtrate by dilute sulphuric acid and the precipitate again filtered off and washed out. Filtrate and washings are combined together, evaporated down and made up to 1 litre and a Kjeldahl nitrogen determination again made. Allowing for the nitrogen given off as ammonia, the difference between this and the previous estimation gives the humin nitrogen II. contained in the alkaline barium magnesia precipitate.

III. Precipitation of Arginine and Histidine.

The solution, which now contains a small quantity of sulphuric acid, is placed in a 5 litre flask, and treated with a hot saturated solution of silver sulphate,[1] which is slowly added, until the solution contains sufficient to give a yellow, not a white, precipitate, when a drop is removed and tested with baryta water in a watch-glass. If, during the process, there be any undissolved silver sulphate at the bottom of the flask, it is dissolved by adding more water before a fresh quantity is added, in order that a yellow precipitate be given in the test drop with baryta. As soon as sufficient silver is present to combine with all the arginine and histidine, it is allowed to cool to 40° C. and then saturated with finely powdered baryta, *i.e.* until some remains undissolved after repeated shaking. The precipitate, which is thus formed

[1] Osborne prefers to use silver nitrate.

and which consists of the silver salts of arginine and histidine, is filtered off and stirred up together with the filter paper in a mortar with baryta, when it is again filtered off and washed with baryta water. The lysine in the filtrate is separated according to VI.

The precipitate of the silver salts of arginine and histidine is suspended in water containing sulphuric acid, and decomposed with hydrogen sulphide. The filtrate from the silver sulphide and barium sulphate, which is thoroughly extracted in the usual manner with boiling water, is evaporated down to remove the hydrogen sulphide and again made up to 1 litre; a Kjeldahl nitrogen determination in 20 c.c. now gives the amount of nitrogen in the substances precipitated by silver and baryta.

IV. Estimation and Isolation of Histidine.

(*a*) The greater portion of the histidine is removed by precipitation with mercuric sulphate. The solution is concentrated to about 250 c.c., sulphuric acid is added till the solution contains 5 per cent. of this acid, and it is treated with a slight excess of mercuric sulphate solution. The precipitate of histidine mercury sulphate is allowed to stand for twelve to twenty-four hours when it is filtered off, washed with 5 per cent. sulphuric acid, suspended in water and decomposed with hydrogen sulphide. The filtrate and washings from the mercuric sulphide which contain the histidine are neutralised with baryta and barium nitrate added until barium sulphate is no longer precipitated. The barium sulphate is filtered off and thoroughly washed. The histidine is then thrown down as silver compound and estimated as under (*b*).

(*b*) The filtrate from the histidine mercuric sulphate is freed from mercury by hydrogen sulphide and from sulphuric acid by neutralising to litmus with baryta and adding barium nitrate as long as a precipitate is formed. Both precipitates are filtered off and washed.

The solution is then concentrated to 300 c.c., acidified with nitric acid, if necessary, and treated with silver nitrate, as before, till a test drop gives a yellow colour with baryta; when this occurs it is exactly neutralised to litmus with baryta and 5 c.c. of a cold saturated solution of baryta are added. If 10 c.c. of the filtered solution when tested with a drop of baryta give a precipitate which indicates that the silver salt of histidine is not completely thrown down, 2 c.c. of saturated baryta solution are added to the main bulk, and this test is repeated

until a test portion remains clear. The precipitate of silver salt of histidine is then filtered off and added to the main portion which has been treated in the same way.

Instead of adding excess of baryta Steudel directs that a suspension of barium carbonate be added to the neutral solution, the solution warmed on the water-bath and then raised to the boiling-point. After cooling, the histidine silver salt is filtered off and washed with baryta till free from nitric acid. The filtrate and washings are treated as in V. for arginine.

The two precipitates of the silver salt of histidine are suspended and heated in water to which sulphuric acid is added until the reaction is acid and decomposed with hydrogen sulphide. Excess of hydrogen sulphide is removed by boiling and the silver sulphide is filtered off and washed. The solution and washings are concentrated and made up to 250 c.c. A nitrogen estimation in 20-25 c.c. by Kjeldahl's method gives the amount of histidine.

The estimation of histidine can be carried out entirely as described under (*b*).

From the remainder of the solution the histidine may be isolated as hydrochloride or as picrolonate :—

1. *As Hydrochloride.*—The solution is made alkaline with baryta, the barium sulphate formed is filtered off, excess of baryta is removed by carbon dioxide, and the whole is evaporated to dryness. The residue is extracted with boiling water and to the solution, filtered from barium carbonate, hydrochloric acid is added. Histidine dichloride, $C_6H_9N_3O_2 . 2HCl$, is obtained on evaporating down. The yield is 75-80 per cent. of the histidine estimated by the Kjeldahl determination.

2. *As Picrolonate.*—The excess of sulphuric acid is removed by treating the hot solution with excess of baryta, and excess of the latter is removed by carbon dioxide; it is then evaporated down and filtered from barium sulphate and carbonate, which are thoroughly washed. The filtrate and washings are evaporated to about 10 c.c., if necessary after the addition of a drop of sulphuric acid to remove the last traces of barium. The necessary quantity of picrolonic acid (calculated from the above Kjeldahl determination and dissolved in a small quantity of alcohol) is added; the precipitate of histidine picrolonate is filtered off after three days, washed with water, dried and weighed. The amount of histidine can be calculated from the formula $C_6H_9N_3O_2 . C_{10}H_8N_4O_5$; it corresponds very closely with the amount calculated from the Kjeldahl estimation.

V. Estimation and Isolation of Arginine.

The filtrate containing the arginine is saturated with baryta; the precipitate of the silver salt of arginine, so obtained, is filtered off, and precipitate and filter paper are stirred up in a mortar with baryta, filtered off, and the process repeated till the precipitate is free from nitric acid. It is then suspended in water containing a slight excess of sulphuric acid and decomposed with hydrogen sulphide. The filtrate and washings from the precipitate of silver sulphide and barium sulphate are evaporated down and made up to 500 c.c., or 1 litre. The amount of arginine is estimated from the amount of nitrogen determined in 25 to 50 c.c. of this solution by Kjeldahl's method.

The arginine is isolated from the remainder of the solution as nitrate, copper nitrate double salt, or as picrolonate.

1. *As Nitrate.*—The solution is freed from sulphuric acid by baryta, the excess of which is removed by carbon dioxide, and evaporated down. The last traces of baryta are then removed with a drop of sulphuric acid, the solution is neutralised with nitric acid and evaporated to dryness. Arginine nitrate, $C_6H_{14}N_4O_2 . HNO_3 + \frac{1}{2}H_2O$ is obtained as a dry white crystalline mass. The double salt with copper nitrate i then prepared from the nitrate; a yield of 85-90 per cent. is obtained.

2. *As Picrolonate.*—The solution is freed from sulphuric acid as described above, and evaporated down to about 10 c.c. The necessary quantity of picrolonic acid (calculated from the nitrogen determination and dissolved in a small volume of hot alcohol) is then added; the yellow crystals of picrolonate are filtered off after a few days, washed with a small quantity of water and dried at 110°. The yield of arginine calculated from the picrolonate, $C_6H_{14}N_4O_2 . C_{10}H_8N_4O_5$, which loses its one molecule of water of crystallisation at 110°, is almost quantitative since the picrolonate has the very slight solubility of 1 part in 1124 parts of water.

VI. Estimation and Isolation of Lysine.

The lysine is contained in the filtrate from the precipitate of the silver salts of arginine and histidine.

The solution is acidified with sulphuric acid and freed from silver by hydrogen sulphide; the filtrate and washings from the precipitate of silver sulphide and barium sulphate, which is treated in the usual manner, are evaporated down to 500 c.c. Sulphuric acid is then added until the content is 5 per cent., and the lysine is precipitated by not too large an excess of phosphotungstic acid. This is added until a portion of the clear liquid on the further addition of the reagent remains clear for ten seconds. After twenty-four hours the precipitate of lysine phosphotungstate is filtered off by suction and washed with 5 per cent. sulphuric acid by stirring up in a mortar. After making up the filtrate and washings to a definite volume an estimation of the substances not precipitated may be made in an aliquot part by the Kjeldahl method.

The lysine phosphotungstate is made into a uniform suspension with water and poured into boiling water. A hot saturated solution of baryta is added until the solution is strongly alkaline and contains excess of baryta. The precipitate of barium phosphotungstate, which is formed, is filtered off and boiled out several times with baryta and then with water. The alkaline solution is freed from bartya by means of carbon dioxide, concentrated, filtered, and evaporated on the water-bath nearly to dryness. Water is then added, the barium carbonate filtered off and washed, and the solution once more evaporated, after which it is made up to a definite volume and the lysine estimated in an aliquot portion by Kjeldahl's method.

The lysine is separated from the remainder of the solution as picrate. The solution is evaporated down in a porcelain basin to dryness, and a small quantity of alcohol is added to the sticky residue. It is then treated with a saturated solution of picric acid in alcohol until no further precipitation of picrate occurs. After twenty-four hours this precipitate is filtered off and washed with a small quantity of absolute alcohol; it is then recrystallised by solution in boiling water, filtering if necessary, and evaporating to a small volume, when lysine picrate, $C_6H_4N_2O_2 . C_6H_2(NO_2)_3OH$, crystallises in needles on cooling; these are filtered off, washed with alcohol, dried and weighed.

The last portions of the lysine in the mother liquor from the picrate can be obtained by acidifying with sulphuric acid, extracting the picric acid with ether, precipitating as phosphotungstate, and repeating the above process for obtaining lysine picrate.

THE RESULTS OF THE ANALYSIS.

A.

Inspection of the results of analysis, which are tabulated on pages 53-62, shows that there is a considerable deficit in the sum of the amino acids composing the protein molecule.

The best analyses are those of the protamines, of the silk-fibroin of spider's silk, and the gliadin of wheat; in these some 80-90 per cent. of the protein is accounted for; in most cases the sum of the figures only reaches 50-70 per cent. and in the other cases complete analyses do not exist. The deficiencies are due almost entirely to losses incurred in isolating and purifying the amino acids rather than to the presence of new units, for no new constituent of the protein molecule has been noted since 1904, when Fischer described diaminotrioxydodecanic acid; this compound has not been found in any other protein than caseinogen, and Osborne with his collaborators in spite of a very careful analysis of this substance failed to find it.

A careful inquiry where the loss occurs has been made by Osborne in conjunction with Leavenworth and Brautlecht and with Jones, and by Abderhalden, who estimates the nitrogen at each stage in the process for isolating the monoamino acids.

Osborne, Leavenworth and Brautlecht have proved that the loss does not fall upon the diamino acids. They are convinced that the Kossel, Kutscher, Patten, Steudel, and Weiss method is extremely satisfactory, for they have been able to recover from 80-90 per cent. of the diamino acids in a pure state. They consider that no other diamino acid than the three hexone bases is present in most proteins; Fischer and Abderhalden's diaminotrioxydodecanic acid in caseinogen may be an exception; this protein seems to be the most complex in the number of units which it contains.

The loss therefore occurs in the isolation and estimation of the monoamino acids. Fischer pointed out, when he first described his ester method, that the values were not to be regarded as quantitative. The fact that all the figures given are those of the amount of the *pure* dry compound isolated is sufficient evidence that the total quantity of products is not accounted for.

The sources of loss in the several steps of the long process have re-

ceived special attention from Osborne and Jones, who are confirmed in their observations by Abderhalden.

1. *Hydrolysis.*—In many cases the hydrolysis of the proteins may not have been complete. Some proteins are very difficult to bring into solution in the concentrated hydrochloric acid, and portions may adhere to the sides of the flask and may therefore not be hydrolysed. Even if there be apparent total solution a small amount may escape hydrolysis by becoming enclosed in the "humin" which is formed to a greater or lesser extent. The insoluble material should be filtered off, washed, and tested with the biuret reaction. The absence of the biuret reaction is not an absolute criterion that hydrolysis is complete, for many polypeptides do not show it and are very resistant to hydrolysis. The residue, if large, should therefore be hydrolysed again. Complete hydrolysis should be tested for by Van Slyke's amino-group method. Osborne has found that hydrolysis is sometimes only complete after boiling for two to five days.

Sulphur-containing substances and sometimes sulphur have been found in the reflux condenser, and the smell of iodoform has been noticed in the hydrolysis of spongin.

2. *Formation of Humin.*—Nearly all proteins on hydrolysis yield an insoluble brownish-black residue. This is probably a mixture of secondary products formed from tryptophane, histidine, and carbohydrate; since zein, which contains no tryptophane, no carbohydrate and only a small quantity of histidine, yields very little humin. The loss of products is considerably greater than the quantity of "humin," which generally amounts to 1-2 per cent. of the protein. A loss, which cannot be estimated, is represented by the soluble brown pigment which colours the solution.

3. *Separation of Glutamic acid Hydrochloride.*—The quantity of glutamic acid precipitated as hydrochloride never represents the total quantity present in the protein. The amount precipitated depends very largely on conditions. The precipitate usually contains ammonium chloride; this is removed by boiling with baryta, and if the baryta be removed with carbon dioxide the barium carbonate may contain barium glutamate, which is very insoluble, and consequently there is loss of this constituent. The remainder of the glutamic acid is recovered with the esters.

4. *Esterification.*—The loss in this process is not great, especially if it be repeated. Loss is chiefly due to hydrolysis of the esters

when the water is removed by evaporation *in vacuo*. Phelps and Tillotson's method seems to be preferable.

5. *Separation of Glycine Ester Hydrochloride.*—The glycine is never completely isolated as ester hydrochloride; the remainder is obtained as ester.

6. *Extraction of Esters.*—Loss always occurs in this part of the process, but is covered when the process is repeated. The loss is largely mechanical and cannot be avoided. A small quantity of organic matter is retained by the sodium sulphate used for drying the esters.

7. *Distillation of Esters.*—(*a*) In distilling off the ether, especially if its volume be large, a considerable quantity of esters distils at the same time. Two receivers should be used and the esters extracted from the distillate.

(*b*) Decomposition of the esters occurs during the distillation, and a more or less large residue represents the loss. The products can be recovered, if necessary. Abderhalden has found that in the process up to this stage 25 per cent. of glutamic acid and 40 per cent. of aspartic acid are lost; some of the glutamic acid is lost by conversion into pyrrolidone carboxylic acid. The loss is greatly diminished if the distillation of fraction IV. be omitted. Osborne and Jones find that the separation of the esters in this fraction is not more troublesome than when they are distilled.

8. *Separation of the Individual Amino Acids.*—The greatest loss occurs here as none of the methods of isolating the compounds are perfect.

(*a*) *Proline.*—The quantity extracted by alcohol is much greater than that obtained in a pure crystalline state and reckoned as proline. Estimation by Van Slyke's method gives the actual amount.

(*b*) *Valine, Leucine, Isoleucine.*—The figures given represent the quantity of substance isolated in a pure state. The leucine figures are really those for leucine + isoleucine.

(*c*) *Glycine and Alanine.*—Nearly all the glycine can be obtained, but the actual amount of alanine is much greater.

(*d*) *Glutamic Acid.*—Probably the figures given for this amino acid most nearly approach the real content of the protein in this constituent.

(*e*) *Aspartic Acid and Serine.*—The figures are much too low, as the method of separation is extremely unsatisfactory.

9. *Oxyproline.*—The amount of this substance in the protein is greater than the quantities which have been isolated. The method for its isolation is so laborious that data are only available for a few proteins.

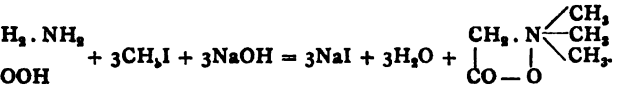
10. *Tyrosine and Cystine.*—The data for tyrosine most probably represent the content of the protein in this unit very closely.

The data for cystine are unsatisfactory; in many cases the data are calculated from the sulphur content of the protein; in the other cases they are those from pure isolated cystine. More satisfactory data are given by Van Slyke's amino method (p. 78).

11. *Tryptophane.*—A large amount of protein is required for the isolation and estimation of tryptophane; it is on this account most probably that so few data exist. The isolation is easier the larger the quantity of material. Tryptophane might be isolated first in most cases and the remainder of the products isolated subsequently.

Osborne, allowing for all losses, calculates that from 41-82 per cent. can actually be recovered. In the cases of zein and gliadin 86 and 68 per cent. respectively are known; in the case of vignin only 5 per cent. more has to be accounted for. The losses from the esters are computed to be 50 per cent. of the alanine, serine, aspartic acid; 30 per cent. of the valine, proline, glutamic acid, phenylalanine; 20 per cent. of the leucine: only 50 per cent. of the cystine and tryptophane may be accounted for.

A method which may prove of some service in separating these various mixtures was described by Engeland in 1907 and 1910. The amino acids are methylated in alkaline solution with methyl iodide and converted into their betaines:—



$$\begin{matrix} CH_2.NH_2 \\ | \\ COOH \end{matrix} + 3CH_3I + 3NaOH = 3NaI + 3H_2O + \begin{matrix} CH_2.N(CH_3)_2 \\ | \\ CO-O \end{matrix}$$

These products are separated by means of their double salts with mercuric chloride, gold chloride and platinum chloride. The details have still to be worked out.

B.

The data in our possession show definitely that the various proteins are composed of the same units; in some cases certain are missing and in other cases one or more units are present in very much larger amount. These differences on the whole confirm our classification of the proteins on physical properties. No great differences are noticeable between the members of any single group except in the case of the scleroproteins. Although two proteins in any group may contain the same amount of any unit we cannot say that they are identical. Even if they contained the same amount of all the units they might still be different, for the arrangement of the units in the molecule may not be the same. The following brief particulars may be noted:—

Protamines.

The protamines are built up almost exclusively of diamino acids, salmine containing over 80 per cent. of arginine. Only small amounts of monoamino acids are present in them; the actual monoamino acid present is very curious, and still more curious is its quantity, namely, 7·8 per cent. of serine and 11 per cent. of proline in salmine, and 8·3 per cent. of tyrosine in cyclopterine. Another feature of salmine is the presence of 87 per cent. of arginine and the absence of lysine and histidine; these peculiarities recur in clupeine and cyclopterine. It is possible that the monoamino acids may be due to impurity, for only at maturity is fish sperm made up of protamine and nucleic acid, whereas at other times histone takes the place of protamine, and histones contain less diamino acids. Kossel and Dakin's analysis of salmine appears to show us a quantitative result.

The presence of diamino acids in all proteins led Kossel to suppose that there was a protamine nucleus (*i.e.* of diamino acids) in all proteins. The more recent work, especially that of Osborne and his collaborators on the gliadins, where the diamino acids are present in such small amounts, though it supports the theory, yet suggests that proteins may exist in which a protamine nucleus is absent, more especially if the view of Emil Fischer be taken that all the proteins we know, even the crystalline ones, are still mixtures of several proteins. The isolation from proteins of complexes containing only diamino acids will be the only proof of a protamine nucleus in a protein molecule.

Histones.

Histones are distinguished from protamines in their smaller content of diamino acids, namely, about 30 per cent. Only in the case of thymus-histone has an estimation been made of the monoamino acids. Histones are supposed to be intermediate compounds between protamines and other proteins, and this supposition is confirmed by the results of analysis.

The protein constituent—globin—of hæmoglobin has always been regarded as a histone, but the presence of only 20 per cent. of diamino acids is against this supposition. Further, the principal diamino acid is histidine, whereas in the other histones it is arginine. It should be noted that hæmoglobin contains a considerably greater amount of histidine than the other proteins. The high content in histidine appears to be a peculiarity of the hæmoglobins; it may be connected with the origin of the red blood corpuscles from nucleated corpuscles since the glyoxaline ring contained in histidine is also contained in the purine bases, which are present in nucleic acid. Comparative data of the hæmoglobin of different animals by Abderhalden and Medigreceanu are at present only available for the red-blood corpuscles; those of the horse contain 5·3 per cent., of the hen 2·8 per cent., of the duck 2·5 per cent., of the goose 3·6 per cent.

Albumins and Globulins.

Albumins contain no glycine, whereas globulins contain this amino acid; they show no other striking differences. Their differentiation on physical grounds is thus scarcely borne out by analysis, and their interconversion, which has been described, may be possible.

The most recent results for crystallised egg-albumin by Osborne, Jones and Leavenworth confirm the earlier ones by Abderhalden and Pregl. They show that workers in different parts obtain very similar results with Fischer's ester method.

The comparative data by Abderhalden and Slavu, both of serum albumin and serum globulin, with regard to their content in glycine, tyrosine, and glutamic acid would incline one to believe that both the serum albumins and the serum globulins of different origin were of the same composition.

Chapman and Petrie have determined that egg-white contains 2·4 per cent. of arginine, 3·2 per cent. of lysine, and 0·7 per cent. of histidine, data which were required for experimental work on nutrition.

Hopkins and Savory's thorough investigation of the Bence-Jones protein in 1911, in which they showed that its peculiar physical properties were due to the conditions under which it was examined, and that its chemical composition differed so distinctly from that of the proteoses, brings this protein into the class of coagulable proteins as a globulin. The Bence-Jones protein is characterised chemically by a high content of the aromatic amino acids; the combined values for phenylalanine and tyrosine are higher than those for any other blood or tissue protein. Both physically and chemically this protein seems to stand in a class by itself.

The Vegetable Proteins.

The vegetable proteins show no great difference from the animal proteins in regard to the number of amino acids which they contain in their molecule. The most noticeable features are their high content in glutamic acid and in arginine. Their ammonia content is also high. This is probably connected with the large amount of the dibasic glutamic acid and is in harmony with the occurrence of asparagine and glutamine in growing seedlings.

Albumins.

Only two vegetable albumins have so far been analysed, the legumelin of the pea and the leucosin of wheat. The resemblance in their composition extends not only to the general proportions of the amino acids, but also to the quantity isolated. Leucosin occurs in the embryo of wheat; it is not possible to locate legumelin in any particular part of the seed, but, from analogy, it may be supposed that both these proteins are constituents of the physiologically active tissues rather than a constituent of the reserve food-stuff for the embryo. Legumelin is quite different in composition from legumin and vicilin, two other proteins contained in the pea. These albumins show a resemblance in their composition to the animal albumins.

Globulins.

The globulins, which can be prepared in a crystalline state, have all a very similar composition. Excelsin contains the greatest amount of arginine and edestin of glutamic acid. These proteins form the best source of arginine. Their content in glutamic acid is about half the content of the gliadins in this amino acid.

No great difference is to be noted between the crystalline globulins and the other vegetable globulins, except in the proportion of arginine which is distinctly less in most of the non-crystalline ones.

The legumins of the pea and vetch show no real difference in their physical properties and elementary composition, but the analysis shows that differences do exist, especially in the data for lysine and histidine. Analysis has also shown that vignin differs from the other legumins.

The vicilin of the pea contains less sulphur (0·1-0·2 per cent.) than any other protein; its analysis shows that it is distinct from legumin. It contains no glycine and more glutamic acid than legumin. A similar protein does not exist in the vetch.

Amandin contains 19 per cent. of nitrogen; the high content in arginine and ammonia serve to explain this high figure.

The high figure for valine in Foreman's analysis of the protein of linseed is remarkable.

Gliadins and Glutelins.

There is a very marked difference in the composition of the two proteins found in cereals. Those amino acids, which are absent in the alcohol-soluble protein, are present in the other protein, which is soluble in dilute alkali. The mixture (gluten) of these proteins in the grain, therefore, gives all the amino acids present in other proteins.

The gliadins are very like one another in composition. They are distinguished from other proteins by their high content in glutamic acid, proline and ammonia, their low content in arginine and histidine, and the absence of lysine. On account of the high content in proline and ammonia Osborne suggested the name of prolamines for this group; the group name—gliadins—has been preferred by the British workers.

Wheat-gliadin and rye-gliadin show no great differences, and it seems probable that they are the same protein. Zein of maize differs from them in containing no glycine and no tryptophane and also in con-

taining more leucine and less glutamic acid. Hordein contains more proline than any other protein. Wheat-glutenin and the other glutelins seem to contain all the amino acids which have been isolated. The analysis of rice by the Japanese workers is for the whole grain and not for the isolated protein; they found that the husk of the grain also contained protein, and that the proportions of the constituent amino acids were different.

Phosphoproteins.

There is no striking peculiarity noticeable in the analyses of the phosphoproteins. If we disregard the small quantity of glycine found in vitellin by Abderhalden and Hunter who used the commercial product in their investigation we must note the absence of this amino acid in the phosphoproteins. Apparently, from the results obtained by numerous workers, glycine is the only amino acid which can be synthesised by the animal body from other products; if these phosphoproteins, especially vitellin, really contain no glycine a further proof is given of its synthesis by animals. Abderhalden and Kempe in their experiments on the synthesis of amino acids in the chick could not detect any differences in the amounts of glycine, tyrosine, and glutamic acid at different periods of development.

The analysis of caseinogen by Osborne and Guest is the most recent, and the data given are the highest which have been observed by them and other workers. On account of the importance of this protein in nutrition it is very necessary to have as thorough an analysis as possible. The latest analysis has increased our knowledge of the constituent amino acids by about 15 per cent. Some 30 per cent. of the protein still remains unaccounted for.

The caseinogens of cow's, goat's, and human milk appear to have the same composition.

Vitellin, which has been analysed by four sets of investigators, has given very different results. The values of Hugounenq and of Levene and Alsberg do not correspond with the values of Abderhalden and Hunter or of Osborne and Jones. The values by the latter workers are the most recent and are probably the most accurate. The phosphoproteins in the eggs of fish (ichthulin) and the frog have also been analysed by Hugounenq and his assistants.

The Scleroproteins.

The scleroproteins, which in their physical properties comprise a heterogeneous collection of proteins, give on hydrolysis, as would be expected, results which support their classification.

Of the proteins in this group those of silk have been most thoroughly investigated. Silk is a mixture of two proteins—silk-fibroin and silk-gelatin; the latter is extracted from raw silk by boiling out with water under pressure when it loses 15-20 per cent. in weight; the insoluble portion which has the structure of the original silk forms the silk-fibroin.

Both silk-fibroin and silk-gelatin were analysed by Fischer and by Fischer and Skita when the ester method was first introduced. Silk-fibroin is composed of practically only three amino acids, glycine, alanine, and tyrosine, and is probably the simplest protein known. It contains more tyrosine than any other protein except that of the carapace of the tortoise and is the best source of tyrosine. Silk-fibroin differs very markedly in composition from silk-gelatin; this substance contains more serine than any other protein.

The composition of silk-fibroin and silk-gelatin from different sources is under investigation by Abderhalden and his pupils. The present data show that the composition of the various silk-fibroins is fairly similar, although many differences can be noted. The New Chwang, Schantung, and Chefoo varieties are rather peculiar in leaving a somewhat large residue after hydrolysis, which seems to be connected with the food-stuff of the silk-worms. The New Chwang and Schantung worms are fed on oak leaves. The Canton and Bengal silks are most like the Italian; Indian Tussore silk contains a considerably smaller amount of glycine. No striking differences are to be noted in the various silk-gelatins. Abderhalden and Dean and Abderhalden and Weichardt have tried to ascertain whether the amino acids composing the silk are elaborated at the moment of spinning or whether they are selected out of the protein material by the spinning gland. Since the composition of the moth and cocoon together is about the same as the silk-worm, it seems most likely that the spinning gland selects the constituents in making the silk and does not synthesise them from other products. Whether the silk-worm can synthesise tyrosine and other amino acids still remains for decision when the analysis of the food has been accomplished.

The silk of other arthropods has also been examined. The silk-fibroin from spider's silk, except for its high content in glutamic acid

closely resembles that of the silk-worm. The silks examined by Suzuki, Yoshimura, and Inouye were distinctly different. The material spun by *Oeceticus* of the family Psychidæ in order to unite the bits of wood together with which it builds its house contained no tyrosine, but otherwise resembled silk-fibroin. The absence of tyrosine brings out a resemblance to ovokeratin.

Gelatin contains no tryptophane, cystine, or tyrosine, but it contains more glycine than any other protein, except elastin and tortoise-shell. It also contains a large amount of proline. This protein appears to have no similarity to silk-gelatin, which contains so much serine. The protein of the carapace of the tortoise is peculiar in containing 13·6 per cent. of tyrosine; silk-fibroin had, until this analysis was performed, been regarded as the protein with most tyrosine in its composition. Whalebone, however, does not show any particular feature.

Spongin resembles gelatin in its high content of glycine, but differs in its content of glutamic acid.

Elastin seems to be made up almost entirely of glycine and leucine; like gelatin, it contains no tyrosine, but it differs from gelatin in containing more phenylalanine.

The keratins are distinguished by containing more cystine than any other protein; in human hair cystine exists to the extent of about 14 per cent., in other keratins its amount varies from 2-8 per cent. (Mörner, Buchtala). Tyrosine is also present in fair quantities, and the amounts of leucine and of glutamic acid are high. Comparative investigations by Abderhalden and Fuchs show that the glutamic acid content of the keratin of ox horn is less in older animals than in younger.

Conjugated and other Proteins.

The only analysis which we possess of a glucoprotein—pseudomucin—was carried out before the ester method had become of general use. Pregl with the small quantity of material available could only perform a qualitative analysis.

A large variety of proteins which cannot be included in any of the above groups have been examined, including micro-organisms and the muscle of Egyptian mummies. The qualitative data given by Abderhalden and Rona for *Aspergillus niger* are of special interest, as this mould was grown on particular nutrient solutions and in each case the same amino acids were synthesised.

The data for the muscle of the Egyptian mummy are interesting as showing that the muscle substance is preserved from decomposition by the process of embalming. Autolysis of the muscle occurs, as amino acids could be extracted from the tissue by water.

The analysis of tumours by the ester method may perhaps throw some light on cancer. Several tumours have been analysed, and they all gave figures approximating to those in the table (p. 62).

By analysing the membrane enveloping the fat particles of milk Abderhalden and Völtz have been able to show that the protein is not caseinogen, but that it is most probably a mixture of proteins, as is generally believed.

The analyses of chicken muscle, fish muscle, scallop muscle, and ox muscle by Osborne and his associates are of extreme importance for the study of the nutritional value of these food-stuffs as compared with one another and with other, especially the vegetable, proteins. The several muscles show a very close resemblance to the vegetable globulins, but they contain less arginine and more lysine than these proteins. The high content in lysine is particularly noticeable; scallop muscle contains the least amount; in the other muscles it is about 7·5 per cent. Fish muscle contains the least amount of glutamic acid; the amounts in the other muscles are very close. The muscles are alike in respect of tyrosine, aspartic acid, arginine, lysine, and histidine, and also phenylalanine, which is slightly more abundant in scallop muscle. Glycine is present in considerable amount in the free state in scallop muscle; otherwise, it is present only in ox muscle to any extent; since syntonin contains very little glycine it probably is derived from the connective tissue in the ox muscle. It is curious that, as we pass from the lower forms of life to the higher, the amounts of glycine, alanine, leucine and proline increase.

The crystalline protein from the juice of *Antiaris toxicaria* examined by Kotake and Knoop is a remarkable protein—probably a complex polypeptide—with 10·6 per cent. of cystine. Further data will no doubt decide the nature of this protein. Only a small quantity of material has been so far available.

Derivatives of Proteins.

Complete analyses of the proteoses—products intermediate between the proteins and amino acids—are being undertaken by Levene in conjunction with Van Slyke and Birchard. The qualitative differences observed by the older workers, Kühne, Chittenden, Neumeister and the analytical differences observed by Pick have not been found. The generally accepted view that heteroalbumose contains more hexone bases than protoalbumose is not confirmed. We may note that heteroalbumose contains more glutamic acid than protoalbumose, and that it contains 1 per cent. more histidine. Skraup and his pupils have made numerous analyses of various intermediate products, all of which seem to contain most of the units. There are differences in the actual amounts but they are insignificant. The examination of the substances termed kyrines by Siegfried leads him to regard them as mixtures. Dennstedt and Hassler believe that in the formation of proteoses the process of oxidation occurs at the same time as hydrolysis.

The analysis of plastein also does not answer the much discussed question whether it represents a synthetical product or a further product of digestion. The figures so nearly resemble those for Witte's peptone that one would be inclined to the view that plastein is still a mixture, which is precipitated under the conditions of the experiment.

The analyses of the proteoses show that they contain all the amino acids originally present in the protein; if a splitting of the large molecule had occurred in such a way that four or five amino acids only were present in each product, the synthetical problem would be easier; we have still no substantial clue as to the order in which the units are combined in the molecule (see Part II.).

PROTAMINES.

	Salmine. (Kossel; Abderhalden; Kossel and Dakin.)	Sturine. (Kossel and Kutscher.)	Clupeine. (Kossel and Kutscher; Kossel and Dakin.)	Scombrine. (Kossel.)	Cyclopterine. (Kossel and Kutscher; Kossel; Morkowin.)	Cyprinine I. (Kossel and Dakin.)	Cyprinine II.
Glycine	...	...	...	...	...	...	...
Alanine	...	...	+	...	...	...	...
Valine	4·3	...	+	...	...	...	+
Leucine	...	...	...	...	...	...	...
Isoleucine	...	...	...	...	...	...	...
Phenylalanine	...	...	...	...	...	...	...
Tyrosine	...	...	...	...	8·3	+ ?	+
Serine	7·8	...	+	...	...	...	...
Cystine	...	...	...	...	...	...	...
Proline	11·0	...	+	...	...	...	...
Oxyproline	...	...	...	...	...	...	...
Aspartic Acid	...	...	...	...	...	...	...
Glutamic Acid	...	...	...	...	...	...	...
Tryptophane	...	...	...	+	+	...	...
Arginine	87·4	58·2	82·2	+	62·5	4·9	+
Lysine	0	12·0	0	0	0	28·8	+
Histidine	0	12·9	0	0	0	0	0
Ammonia	...	...	...	...	...	...	...
Total	110·5	83·1	82·2	...	70·8	33·7	...

HISTONES.

	Globin of Hæmoglobin from Horse's Blood. (Abderhalden.)	Globin of Hæmoglobin from Dog's Blood. (Abderhalden and Baumann.)	Thymus-Histone (Kossel and Kutscher; Kutscher; Abderhalden and Rona.)		Lota-Histone. (Ehrström.)	Gadus-Histone. (Kossel and Kutscher.)
Glycine	...	...	...	0·5	...	...
Alanine	4·2	3·0	...	3·5	...	...
Valine	...	1·0	...	...	...	...
Leucine	29·0	17·5	...	11·8	...	...
Isoleucine	...	...	...	...	...	...
Phenylalanine	4·2	5·0	...	2·2	...	...
Tyrosine	1·3	...	6·4	5·2	...	...
Serine	0·6	...	...	...	...	...
Cystine	0·3	...	...	...	...	...
Proline	2·3	4·5	...	1·5	...	...
Oxyproline	1·0	...	...	...	...	...
Aspartic Acid	4·4	2·5	...	0	...	...
Glutamic Acid	1·7	1·2	3·7	0·5	...	...
Tryptophane	+	...	...	...	...	...
Arginine	5·4	...	14·4	15·5	12·0	15·6
Lysine	4·3	...	7·7	6·9	3·2	8·3
Histidine	11·0	...	1·3	1·5	2·9	2·4
Ammonia	...	...	1·7	...	0·7	0·8
Total	69·7	34·7	35·2	49·1	18·8	27·1

ALBUMINS.

	Egg-albumin. (Abderhalden and Pregl; Mörner; Hugounenq and Galimard.)	Cryst. Egg-albumin. (Osborne, Jones and Leavenworth.)	Serum-albumin from Horse's Blood. (Abderhalden; Mörner.)	Serum-albumin from Goose's Blood. (Abderhalden and Slavu.)	Lact-albumin. (Abderhalden and Pribram.)
Glycine	0	0	0	0	0
Alanine	2·1	2·2	2·7	...	2·5
Valine	...	2·5	...	...	0·9
Leucine	6·1	10·7	20·0	...	19·4
Isoleucine	...	...	...	...	...
Phenylalanine	4·4	5·1	3·1	...	2·4
Tyrosine	1·1	1·8	2·1	2·0	0·9
Serine	...	?	0·6	...	...
Cystine	0·3	?	2·5	...	...
Proline	2·3	3·6	1·0	...	4·0
Oxyproline	...	...	...	...	...
Aspartic Acid	1·5	2·2	3·1	..	1·0
Glutamic Acid	8·0	9·1	7·7	8·1	10·1
Tryptophane	+	+	+	...	...
Arginine	2·1	4·9	...	...	...
Lysine	2·1	3·8	...	...	...
Histidine	0	1·7	...	...	...
Ammonia	...	1·3	...	...	...
Total	30·0	48·9	42·8	10·1	41·2

GLOBULINS.

	Serum-globulin from Horse's Blood. (Abderhalden; Abderhalden and Samuely; Mörner.)	Serum-globulin from Goose's Blood. (Abderhalden and Slavu.)	Fibrin. (Abderhalden and Voitinovici; Mörner.)	Bence-Jones' Protein.	
				(Abderhalden and Rostoski.)	(Hopkins and Savory.)
Glycine	3·5	3·6	3·0	1·7	+
Alanine	2·2	...	3·6	4·5	+
Valine	+	...	1·0	...	5·6
Leucine	18·7	...	15·0	10·6	5·5
Isoleucine	...	...	...	...	1·0
Phenylalanine	3·8	...	2·5	1·5	4·9
Tyrosine	2·5	2·5	3·5	1·7	4·2
Serine	...	...	0·8	...	...
Cystine	1·5	...	1·1	...	0·6
Proline	2·8	...	3·6	1·9	2·7
Oxyproline	...	...	...	...	...
Aspartic Acid	2·5	...	2·0	4·5	2·2
Glutamic Acid	8·5	9·1	10·4	6·0	8·0
Tryptophane	+	...	+	...	0·8
Arginine	...	...	...	...	6·1
Lysine	...	...	...	...	3·7
Histidine	...	...	...	...	0·8
Ammonia	...	...	...	...	...
Total	46·0	15·2	46·5	32·4	46·1

DERIVATIVES OF PROTEINS.

	Syntonin. (Abderhalden and Sasaki; Hart.)	Hetero-albumose. from Syntonin. (Hart.)	Proto-albumose, from Syntonin. (Hart.)	Witte's Peptone. (Levene and Van Slyke.	Plastein. (Levene and Van Slyke.)	Proto-albumose. (Levene, Van Slyke and Birchard.)	Hetero-albumose, from Witte's Peptone. (Levene, Van Slyke and Birchard.)	Hemielastin (Elastose) (Wechsler.)
Glycine	0·5	...	...	0·8	2·2	1·5	0·2	...
Alanine	4·0	...	...	2·8	?	2·5	3·4	...
Valine	0·9	...	...	} 14·7	} 15·6	0·8	3·5	...
Leucine	7·8	...	...			5·8	3·1	...
Isoleucine	...	...	...	...	...	1·6	3·0	...
Phenylalanine	2·5	...	...	2·6	1·0	4·4	2·5	...
Tyrosine	2·2	...	...	3·3	3·0	4·6	3·5	...
Serine	...	...	...	1·2	...	...	...	...
Cystine	...	...	...	...	...	0·7	4·1	...
Proline	3·3	...	...	4·6	2·6	5·0	4·3	...
Oxyproline	...	...	...	0·0	...	...	...	...
Aspartic Acid	0·5	...	...	1·7	2·2	3·0	4·7	...
Glutamic Acid	13·6	...	...	8·2	10·0	0·6	9·5	...
Tryptophane	...	...	...	+	+	...	...	...
Arginine	5·1	8·5	4·6	1·5	2·1	7·7	6·4	1·9
Lysine	3·3	7·1	3·1	2·7	1·4	8·4	4·8	2·5
Histidine	2·7	0·4	3·4	0·8	0·4	2·8	1·8	0·5
Ammonia	0·9	1·0	0·8	...	...	0·9	1·7	0·1
Total	47·3	17·0	11·9	44·9	40·5	50·3	56·5	5·0

CRYSTALLISED VEGETABLE GLOBULINS.

	Edestin from Hemp Seed. (Abderhalden; Kossel and Patten; Schulze and Winterstein; Osborne and Liddle.)			Edestin from Cotton Seed. (Abderhalden and Rostoski.)	Edestin from Sunflower Seed. (Abderhalden and Reinbold.)	Cryst. Globulin from Pumpkin Seed. (Abderhalden and Berghausen.)	Cryst. Globulin from Squash Seed. (Osborne and Clapp; Osborne and Gilbert.)	Excelsin from Brazil Nut. (Osborne and Clapp.)
Glycine	...	3·8	...	1·2	2·5	0·1	0·6	0·6
Alanine	...	3·6	...	4·5	4·5	+	1·9	2·3
Valine	...	+	...	+	0·6	0·7	0·3	1·5
Leucine	...	20·9	...	15·5	12·9	4·7	7·3	8·7
Isoleucine	...	...	...	...	...	...	...	...
Phenylalanine	...	3·1	...	3·9	4·0	2·6	3·3	3·5
Tyrosine	...	2·1	...	2·3	2·0	1·4	3·1	3·1
Serine	...	0·4	...	0·4	0·2	...	...	...
Cystine	...	0·3	...	...	...	...	0·3	...
Proline	...	4·1	...	2·3	2·8	1·7	2·9	3·6
Oxyproline	...	? 2·0	...	...	...	...	...	...
Aspartic Acid	...	4·5	...	2·9	3·2	4·5	3·3	3·8
Glutamic Acid	...	18·8	...	17·2	13·0	13·4	12·4	12·9
Tryptophane	...	+	...	+	+	...	+	+
Arginine	11·2	11·7	14·4	...	...	...	14·4	16·1
Lysine	1·6	1·0	1·7	...	...	...	2·0	1·6
Histidine	1·4	1·1	2·4	...	...	...	2·6	1·5
Ammonia	...	...	...	...	...	...	1·6	1·8
Total	...	77·4	...	50·2	45·7	29·1	56·0	61·0

VEGETABLE GLOBULINS.

	Legumin from Pea. (Osborne and Clapp.)	Legumin from Vetch. (Osborne and Heyl.)	Legumin from White Beans. (Abderhalden and Babkin; Schulze and Winterstein.)	Phaseolin from White Kidney Bean. (Osborne and Clapp.)	Glycinin from Soy Bean. (Osborne and Clapp.)	Vignin from Cow-Pea. (Osborne and Heyl.)
Glycine . .	0·4	0·4	1·0	0·6	1·0	0·0
Alanine . .	2·1	1·2	2·8	1·8	...	1·0
Valine . .	...	1·4	1·0	1·1	0·7	0·4
Leucine . .	8·0	8·8	8·2	9·7	8·5	7·8
Isoleucine .	...	...	...	...	...	...
Phenylalanine	3·8	2·9	2·0	3·3	3·9	5·3
Tyrosine .	1·6	2·4	2·8	2·2	1·9	2·3
Serine . .	0·5	?	...	0·4	...	0
Cystine . .	...	...	...	...	...	...
Proline . .	3·2	4·1	2·3	2·8	3·8	5·3
Oxyproline .	...	...	...	...	...	0
Aspartic Acid .	5·3	3·2	4·0	5·3	3·9	4·0
Glutamic Acid	17·0	18·3	16·3	14·6	19·5	16·9
Tryptophane .	+	+	...	...	...	+
Arginine .	11·7	11·1	4·6	4·9	5·1	7·2
Lysine . .	5·0	4·0	5·1	4·0	2·7	4·3
Histidine .	2·4	2·9	1·1	2·0	1·4	3·1
Ammonia .	2·1	2·1	...	2·1	2·6	2·3
Total .	63·1	62·8	51·2	54·8	55·0	59·9

VEGETABLE GLOBULINS.

	Amandin from Almond. (Osborne and Clapp; Osborne and Gilbert.)	Vicilin from Pea. (Osborne and Heyl.)	Conglutin from Lupine. (Abderhalden and Herrick; Schulze and Winterstein.)	Protein from Linseed. (Foreman.)	Protein from Fir-tree Seed. (Abderhalden and Teruuchi; Schulze and Winterstein.)	Protein from Pinus Koraiensis. (Yoshimura.)
Glycine . .	0·5	0·0	0·8	+	0·6	...
Alanine . .	1·4	0·5	2·5	1·1	1·8	...
Valine . .	0·2	0·2	1·1	12·7	+	...
Leucine .	4·5	9·4	6·8	} 4·0	6·2	11·4
Isoleucine .	...	...	...		...	...
Phenylalanine	2·5	3·8	3·1	4·2	1·2	...
Tyrosine .	1·1	2·4	2·1	0·7	1·7	2·5
Serine . .	?	?	+	+	0·1	...
Cystine . .	...	...	...	...	0·3	...
Proline . .	2·5	4·1	2·6	2·9	2·8	...
Oxyproline .	...	...	...	...	...	...
Aspartic Acid	5·4	5·3	3·0	1·7	1·8	...
Glutamic Acid	23·2	21·4	19·5	11·6	7·8	2·7
Tryptophane	+	+	+	+	+	...
Arginine .	11·9	8·9	6·9	6·1	10·9	7·1
Lysine . .	0·7	5·4	2·1	1·2	0·3	0·9
Histidine .	1·6	2·2	0·7	1·7	0·7	0·5
Ammonia .	3·7	2·0	...	1·9	...	...
Total .	59·4	65·6	51·2	49·8	36·2	25·1

	VEGETABLE ALBUMINS.		GLUTENS.		
	Legumelin from Pea. (Osborne and Heyl.)	Leucosin from Wheat. (Osborne.)	Wheat. (Abderhalden and Malengreau; Kossel and Kutscher.)	Oats. (Avenin.) (Abderhalden and Hämäläinen.)	Maize. (Osborne and Clapp.)
Glycine	0·5	0·9	0·4	1·0	0·3
Alanine	0·9	4·5	0·3	2·5	...
Valine	0·7	0·2	...	1·0	...
Leucine	9·6	11·3	4·1	15·0	6·2
Isoleucine	...	...	...	...	...
Phenylalanine	4·8	3·8	1·0	3·2	1·8
Tyrosine	1·6	3·3	1·9	1·5	3·8
Serine	?	?	...	...	...
Cystine	...	...	...	...	...
Proline	4·0	3·2	4·0	5·4	5·0
Oxyproline	...	...	...	...	...
Aspartic Acid	4·1	3·4	0·7	4·0	0·7
Glutamic Acid	13·0	6·7	24·0	18·4	12·7
Tryptophane	+	+	+	...	+
Arginine	5·5	5·9	4·4	...	7·1
Lysine	3·0	2·8	2·2	...	3·0
Histidine	2·3	2·8	1·2	...	3·0
Ammonia	1·3	1·4	2·5	...	2·1
Total	51·3	50·2	46·7	52·8	45·7

GLIADINS.

	Gliadin, from Wheat. (Abderhalden and Samuely; Kossel and Kutscher; Kutscher.)	Gliadin, from Wheat. (Osborne and Clapp; Osborne and Guest.)	Gliadin, from Rye. (Osborne and Clapp.)	Hordein, from Barley. (Osborne and Clapp.)	Hordein. (Kleinschmitt.)	Zein, from Maize. (Osborne and Clapp; Osborne and Liddle.)	Zein, from Maize. (Langstein; Kutscher; Kossel and Kutscher.)
Glycine	0·7	0·0	0·13	0·0	0·0	0·0	–
Alanine	2·7	2·0	1·33	0·4	1·4	9·8	0·5
Valine	0·4	3·4	...	0·2	1·4	1·9	+
Leucine	6·0	6·6	6·30	5·7	7·0	19·6	11·2
Isoleucine	...	...	...	...	...	...	...
Phenylalanine	2·6	2·4	2·70	5·0	5·5	6·6	7·0
Tyrosine	2·4	1·2	1·19	1·7	4·0	3·6	10·1
Serine	0·2	0·2	0·06	...	0·1	1·0	–
Cystine	...	0·5	...	...	...	...	...
Proline	2·4	13·2	9·82	13·7	5·9	9·0	1·5
Oxyproline	...	...	...	...	...	...	...
Aspartic Acid	1·3	0·6	0·25	...	1·3	1·7	1·0
Glutamic Acid	31·5	43·7	33·81	36·4	41·3	26·2	11·8
Tryptophane	1·0	1·0	+	+	...	0·0	...
Arginine	2·8	3·2	2·22	2·2	3·2	1·6	1·9
Lysine	0·0	0·0	0·00	0·0	0·0	0·0	0·0
Histidine	1·2	0·6	0·39	1·3	0·5	0·8	0·9
Ammonia	4·1	5·2	5·11	4·9	4·4	3·6	2·6
Total	59·3	83·8	64·31	71·5	76·0	85·4	48·5

PHOSPHOPROTEINS.

	Caseinogen, Cow's Milk. (Abderhalden; Fischer; Mörner; Fischer and Abderhalden; Hart.)	Caseinogen, Cow's Milk. (Osborne and Guest.)	Caseinogen, Goat's Milk. (Abderhalden and Schittenhelm.)	Caseinogen, Human Milk. (Abderhalden and Schittenhelm; Abderhalden and Langstein.)	Vitellin. (Abderhalden and Hunter.)	Vitellin. (Levene and Alsberg)	Vitellin. (Hugounenq.)	Vitellin. (Osborne and Jones.)
Glycine	0	0	0	0	1·1	trace	<0·5	0
Alanine	0·9	1·5	1·5	1·2	+	0·2	<0·5	0·8
Valine	1·0	7·2	...	1·3	2·4	...	1·5	1·9
Leucine	10·5	9·4	7·4	8·8	11·0	3·3	6·8	9·9
Isoleucine	...	...	...	...	...	...	...	...
Phenylalanine	3·2	3·2	2·8	2·8	2·8	1·0	0·7	2·6
Tyrosine	4·5	4·5	5·0	4·7	1·6	0·4	2·0	3·4
Serine	0·3	0·5	...	...	–	...	<0·5	...
Cystine	0·1	?	...	...	...	...	...	...
Proline	3·1	6·7	4·6	2·9	3·3	4·0	<0·5	4·2
Oxyproline	0·3	0·3	...	...	...	...	...	...
Aspartic Acid	1·2	1·4	1·2	1·0	0·5	0·6	0·7	2·2
Glutamic Acid	11·0	15·6	12·0	11·0	12·2	1·0	1·0	13·0
Tryptophane	1·5	1·5	+	...	...	...	...	+
Arginine	4·8	3·8	...	...	...	1·2	1·0	7·5
Lysine	5·8	6·0	...	...	...	2·4	1·2	4·8
Histidine	2·6	2·5	...	...	...	trace	2·1	1·9
Ammonia	1·6	1·6	...	...	..	...	1·2	1·3
Total	51·4	66·5	34·5	33·7	34·9	14·1	20·2	53·5

SCLEROPROTEINS.

SILK-FIBROIN.

	Italian. (Fischer and Skita; Fischer.)	Italian. Cocoon. (Roose.)	Chinese. New Chwang. (Abderhalden and Rilliet.)	Chinese. Canton. (Abderhalden and Behrend.)	Chinese. Shantung. (Abderhalden and Brahm.)	Chinese. Niet ngō Tsam. (Abderhalden and Brossa.)	Chinese. Tai-Tsao-Tsam. (Abderhalden and Schmid.)	Chinese. Chefoo. (Abderhalden and Welde.)	Japanese. Cocoon. (Suwa.)	Indian. Bengal. (Abderhalden and Sington.)	Indian. (Abderhalden and Spack.)
Glycine . .	36·0	33·5	19·7	37·5	14·5	24·0	25·2	12·5	35·0	30·5	9·5
Alanine . .	21·0	20·0	23·8	23·5	22·0	18·5	18·2	18·0	22·6	20·0	24·0
Valine . .	0	...	...	...	...	...	...	...	...	...	...
Leucine .	1·5	0·8	1·6	1·5	1·0	1·2	0·9	1·2	0·7	1·2	1·5
Isoleucine .	...	...	...	...	...	...	...	...	...	...	...
Phenylalanine	1·5	1·2	1·2	1·6	1·0	1·0	1·0	1·0	1·3	1·4	0·6
Tyrosine .	10·5	9·0	9·8	9·8	9·7	7·8	7·8	8·5	9·7	10·0	9·2
Serine . .	1·6	1·9	1·0	1·5	1·8	1·5	1·2	1·0	0·7	1·8	2·0
Cystine . .	...	...	...	...	...	...	...	...	...	...	...
Proline . .	+	0·8	1·9	1·0	2·5	1·2	1·0	2·5	0·7	1·0	1·0
Oxyproline .	...	...	...	...	...	...	...	...	...	...	...
Aspartic Acid	+	1·0	2·9	0·8	1·0	2·0	2·1	2·0	1·0	0·8	2·5
Glutamic Acid	0	0·3	1·7	...	1·8	3·0	2·0	2·0	0·1	+	1·0
Tryptophane .	...	...	...	...	...	...	...	...	...	...	...
Arginine .	1·0	...	...	...	...	...	...	...	...	...	...
Lysine . .	+	...	...	...	...	...	...	...	...	...	...
Histidine .	+	...	...	...	...	...	...	...	...	...	...
Ammonia .	...	...	...	...	...	...	...	...	...	...	...
Total .	73·1	68·5	61·6	77·2	55·3	60·2	59·4	48·7	71·8	66·7	51·3

SCLEROPROTEINS.

	Silk-fibroin of Spider. (Fischer.)	Silk of Oecetícus platensis (Berg.). (Abderhalden and Landau.)	Silk of Antheraea Peruyi. (Suzuki, Yoshimura and Inouye.)	Silk of Antheraea Yamamai (Suzuki, Yoshimura and Inouye.)	Silk of Caligula Japonica (Suzuki, Yoshimura and Inouye.)	Silk of Cnethocampa pityocampa. (Abderhalden and Behrend.)
Glycine	35·2	27·1	5·7	6·3	7·7	+
Alanine	23·4	18·8	4·8	7·2	15·3	+
Valine	...	...	...	...	–	...
Leucine	1·8	0·8	1·2	1·3	8·0	...
Isoleucine	...	...	...	...	...	...
Phenylalanine	...	1·8	...	+	+	...
Tyrosine	8·2	...	1·4	2·0	5·5	+
Serine	...	...	...	...	...	...
Cystine	...	...	...	...	...	...
Proline	3·7	3·2	+	+	4·2	...
Oxyproline	...	...	...	...	...	...
Aspartic Acid	...	0·3	1·0	1·0	0·2	...
Glutamic Acid	11·7	2·4	+	0·6	?	...
Tryptophane	...	...	...	...	...	...
Arginine	...	...	3·1	3·8	1·7	...
Lysine	5·2	...	?	7·4	2·4	...
Histidine	...	...	2·7	1·6	1·0	...
Ammonia	1·2	...	0·6	0·8	0·8	...
Total	90·4	54·4	20·5	32·0	46·8	...

SCLEROPROTEINS.

SILK-GELATIN.

	Italian. (Fischer and Skita; Fischer.)	Chinese. Canton. (Abderhalden and Worms.)	Indian. (Strauch.)	Silkworm caterpillar. (Abderhalden and Dean.)	Silkworm moth. (Abderhalden and Weichardt.)
Glycine	0·2	1·2	1·5	10·2	3·5
Alanine	5·0	9·2	9·8	8·7	3·2
Valine	...	?	...	1·7	1·7
Leucine	...	5·0	4·8	4·8	8·5
Isoleucine	...	...	...	...	...
Phenylalanine	...	0·6	0·3	2·4	2·7
Tyrosine	5·0	2·3	1·0	4·3	1·6
Serine	6·6	5·8	5·4	...	...
Cystine	...	...	...	...	...
Proline	...	2·5	3·0	1·5	4·0
Oxyproline	...	...	...	...	...
Aspartic Acid	...	2·5	2·8	1·6	2·7
Glutamic Acid	...	2·0	1·8	3·5	5·7
Tryptophane	...	...	...	...	...
Arginine	4·0	...	...	...	...
Lysine	...	...	...	...	...
Histidine	0	...	...	...	...
Ammonia	...	...	...	...	...
Total	20·8	31·1	30·4	38·7	33·6

SCLEROPROTEINS.

	Gelatin. (Fischer, Levene and Adera; Fischer, Hart, Kossel and Kutscher; Fischer and Boehner.)		Elastin. (Abderhalden and Schittenheim; Schwarz; Kossel and Kutscher.)	Spongin. (Abderhalden and Strauss; Kossel and Kutscher.)	Koilin. (Kneffl-Lenz; Hofmann and Pregl.)	Egg-membrane of Scyllium Stellare. (Pregl.)	Neuro-Keratin. (Argiris.)	Whalebone of North whale. (Abderhalden and Landau.)	Carapace of Tortoise. (Buchtala.)
Glycine	...	16·5	25·8	13·9	1·2	2·6	...	0·8	19·4
Alanine	...	0·8	6·6	...	5·8	3·2	...	6·4	3·0
Valine	...	1·0	1·0	...	...	...	...	9·7	5·2
Leucine	...	2·1	21·4	7·5	} 13·2	5·8	...	3·8	3·3
Isoleucine	...	–	...	...		...	...	...	...
Phenylalanine	...	0·4	3·9	...	2·3	3·3	...	0·5	1·1
Tyrosine	...	0	0·4	0	5·4	10·6	4·6	5·7	13·6
Serine	...	0·4	...	...	...	...	...	1·0	...
Cystine	...	–	...	...	0·8	?	1·5	4·2	5·2
Proline	...	7·7	1·7	6·3	5·5	4·4	...	2·6	...
Oxyproline	...	3·0	...	...	...	...	...	...	...
Aspartic Acid	...	0·6	+	4·7	2·3	2·3	...	2·6	...
Glutamic Acid	...	0·9	0·8	18·1	5·2	7·2	...	8·9	0
Tryptophane	...	0	...	...	...	+	...	...	...
Arginine	9·3	7·6	0·3	...	3·6	3·2	2·2	...	...
Lysine	5·0	2·8	...	...	1·7	3·7	2·7	...	...
Histidine	...	0·4	...	...	0·1	1·7	0·8	...	...
Ammonia	...	0·4	...	...	...	...	...	...	...
Total	...	44·6	61·9	50·5	47·1	48·0	11·8	46·2	50·8

SCLEROPROTEINS.

	Keratin, from Ox Horn. (Fischer and Dörpinghaus; Mörner.)	Keratin, from Sheep's Horn. (Abderhalden and Voitinovici.)	Keratin, from Sheep's Wool. (Abderhalden and Voitinovici.)	Keratin, from Horse Hair. (Abderhalden and Wells; Argiris; Buchtala.)	Keratin, from Goose Feathers. (Abderhalden and Le Count.)	Keratin, from Egg-membrane. (Abderhalden and Ebstein; Mörner.)	Keratin, from Egg-membrane of Testudo Græca. (Abderhalden and Strauss.)	Ichthylepidin, from Fish Scales. (Abderhalden and Voitinovici.)
Glycine	0·4	0·5	0·6	4·7	2·6	3·9	+	5·7
Alanine	1·2	1·6	4·4	1·5	1·8	3·5	+ ?	3·1
Valine	5·7	4·5	2·8	0·9	0·5	1·1	...	...
Leucine	18·3	15·3	11·5	7·1	8·0	7·4	...	15·1
Isoleucine	...	...	...	...	...	...	...	...
Phenylalanine	3·0	1·9	...	0	0	...	+ ?	...
Tyrosine	4·6	3·6	2·9	3·2	3·6	...	...	1·0
Serine	0·7	1·1	0·1	0·6	0·4	...	...	...
Cystine	6·8	7·5	7·3	8·0	...	7·6	...	...
Proline	3·6	3·7	4·4	3·4	3·5	4·0	11·8 ?	6·7
Oxyproline	...	...	...	...	...	...	...	...
Aspartic Acid	2·5	2·5	2·3	0·3	1·1	1·1	1·8 ?	1·2
Glutamic Acid	3·0	17·2	12·9	3·7	2·3	8·1	3·0 ?	9·2
Tryptophane	...	...	...	...	...	...	...	...
Arginine	2·3	2·7	...	4·5	...	...	...	...
Lysine	...	0·2	...	1·1	...	...	...	...
Histidine	...	...	...	0·6	...	...	...	...
Ammonia	...	...	...	...	...	...	...	...
Total	52·1	62·3	49·2	39·6	23·8	36·7	16·6 ?	42·0

VARIOUS PROTEINS.

	Pseudomucin. (Otori.)	Paramucin. (Pregl.)	Phosphorescent Infusoria. (Emmerling.)	Aspergillus Niger. (Abderhalden and Rona.)	Protein in Urine. (Abderhalden and Pregl.)	Muscle of Egyptian Mummies. (Abderhalden and Brahm.)	Tumours. (Abderhalden and Medigreceanu.)
Glycine	0·2	...	15·9	+	+	+	2
Alanine	...	+	2·4	+	+	+	...
Valine	...	...	...	...	...	+	...
Leucine	4·7	+	0·4	+	+	+	...
Isoleucine	...	...	...	...	...	...	...
Phenylalanine	...	+	...	...	...	+	...
Tyrosine	1·1	+	0·6	...	...	+	2
Serine	...	...	...	...	...	...	...
Cystine	...	...	...	...	...	...	...
Proline	...	+	4·6	...	...	+	...
Oxyproline	...	...	...	...	...	...	...
Aspartic Acid	+	+	0·2	+	+	+	...
Glutamic Acid	0·6	+	...	+	+	+	12
Tryptophane	...	+	...	...	...	...	...
Arginine	0·8	+	1·7	...	...	+	...
Lysine	2·7	+	0·2	...	...	+	...
Histidine	...	+	3·5	...	...	+	...
Ammonia	3·2	...	...	...	...	...	...
Total	13·3	...	29·5	...	...	...	16

VARIOUS PROTEINS.

	Chicken Muscle. (Osborne and Heyl.)	Fish (halibut) Muscle. (Osborne and Heyl.)	Scallop Muscle. (Osborne and Jones.)	Ox Muscle. (Osborne and Jones.)	Cryst. Protein from Antiaris Toxicaria. (Kotake and Knoop.)	Placenta. (Koelker and Siemons.)	Amyloid. (Neuberg.)
Glycine	0·7	0·0	0·0	2·1	0·3	0·6	0·8
Alanine	2·3	?	...	3·7	9·0	?	...
Valine	?	0·8	...	0·8	2·4	6·7	...
Leucine	11·2	10·4	8·8	11·7	...	4·4	22·2
Isoleucine	...	...	...	...	...	...	
Phenylalanine	3·6	3·1	4·9	3·2	...	2·2	...
Tyrosine	2·2	2·4	2·0	2·2	2·7	1·7	4·0
Serine	?	?	?	?	...	...	...
Cystine	...	...	...	...	10·6	...	...
Proline	4·8	3·2	2·3	5·8	4·6	1·7	3·1
Oxyproline	...	...	...	...	...	...	...
Aspartic Acid	3·2	2·8	3·5	4·5	...	2·1	...
Glutamic Acid	16·5	10·1	14·9	15·5	...	2·8	3·8
Tryptophane	+	+	+	+	...	+	...
Arginine	6·5	6·4	7·4	7·5	...	4·3	13·9
Lysine	7·3	7·5	5·8	7·6	1·4	3·5	11·6
Histidine	2·5	2·6	2·0	1·8	...	0·3	0
Ammonia	1·7	1·4	1·1	1·1	...	1·3	...
Total	62·5	50·7	52·7	67·5	31·0	31·6	59·4

DIFFERENTIATION OF THE PROTEINS BY THE DISTRIBUTION OF THE VARIOUS KINDS OF NITROGEN.

Since a complete analysis of a protein is still an impossibility, owing to the unsatisfactory methods for isolating and estimating the several monoamino acids, the proteins cannot yet be differentiated by means of their chemical composition.

It has been proved by Osborne, Leavenworth and Brautlecht that the methods for estimating the ammonia content and the diamino acid (the three hexone bases) content of a protein are almost perfect. We can therefore differentiate proteins by their content in these four products.

The monoamino acids and tryptophane all contain an amino group: proline, oxyproline and tryptophane contain a heterocyclic ring: cystine contains sulphur. These differences can be made use of for a further differentiation as was shown by Van Slyke in 1911. We can ascertain the following particulars:—

I. Amide nitrogen (ammonia).

II.
- Diamino nitrogen
  - *a* cystine, *b* lysine — contain only amino N. Sulphur content gives cystine. Lysine by difference
  - and
  - *c* arginine, *d* histidine — arginine contains $\frac{3}{4}$ of its N, histidine „ $\frac{1}{2}$ of its N (in non-amino form.) — Arginine evolves half its N as NH_2 by boiling with alkali. Histidine by difference.
- Cystine nitrogen

III.
- Monoamino nitrogen
  - *a*: glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, aspartic acid, glutamic acid, tryptophane ($\frac{1}{2}$)
- Non-amino nitrogen
  - *b*: proline, oxyproline, tryptophane ($\frac{1}{2}$)

i.e., seven data out of the possible eighteen. If we regard the data for tyrosine as almost accurate, we have still one more value of service for the chemical differentiation of the proteins. It is unfortunate that we cannot yet measure the trypotophane content of a protein, especially as this unit is so readily detected by means of its colour reactions.

A. Distribution of the Nitrogen in Three Groups.

The differentiation of proteins by the estimation of the various groups of the units was first attempted in Hofmeister's laboratory by Hausmann in 1899, who estimated amide nitrogen, diamino nitrogen and monoamino nitrogen. The protein (1 gramme) was hydrolysed with 20 c.c. of concentrated hydrochloric acid by boiling for five hours under a reflux condenser; the solution was diluted and distilled with excess of magnesia, and the ammonia, which was liberated, was collected in excess of standard acid; the solution was then acidified with acid and precipitated with phosphotungstic acid; after twenty-four hours the precipitate was filtered off, washed with phosphotungstic acid, dissolved in alkali and the nitrogen estimated in an aliquot portion by Kjeldahl's method; the filtrate was made up to a definite volume and nitrogen estimated in an aliquot portion.

Numerous objections to the accuracy of the data were raised. Henderson maintained that the amount of amide nitrogen varied according to the strength of the acid employed in the hydrolysis and the time of hydrolysis; Kutscher, and also Chittenden and Eustis, showed that the precipitation of the diamino acids was not complete, and Schulze and Winterstein found that certain monoamino acids, *e.g.*, phenylalanine, were precipitated by phosphotungstic acid under certain conditions. Hart preferred barium carbonate to magnesia for distilling off the ammonia.

Osborne and Harris, Gümbel, and also Rothera, critically examined the various objections. The amount of amide nitrogen was not found to vary as Henderson stated; if similar conditions are always maintained in the precipitation with phosphotungstic acid most valuable comparative results can be obtained; the errors of incomplete precipitation of the diamino acids and precipitation of monoamino acids almost compensate each other.

Adopting Gümbel's suggestion of distilling off the ammonia *in vacuo* at 40° and Osborne and Harris' procedure, the process may be carried out as follows:—

1. About 1 grm. of protein is boiled with about 100 c.c. of 20 per cent. hydrochloric acid in a 500 c.c. round bottom flask under a reflux condenser until the solution no longer gives the biuret reaction, usually from seven to ten hours. It is then evaporated *in vacuo* at 40° to a volume of 2-3 c.c.; the greater portion of the hydrochloric acid is thus removed.

2. About 300 c.c. of water is then placed in the flask and a cream of magnesia, which has been freed from every trace of ammonia by long boiling, is added until in slight, but distinct, excess. The mixture is distilled *in vacuo* at 40° and the distillate collected in excess of standard acid; about half the liquid should be distilled. Titration of the standard acid gives the amount of amide nitrogen.

3. The remainder of the solution is filtered through a nitrogen-free paper and the residue, thus collected, washed thoroughly with water. The nitrogen in this precipitate is estimated by Kjeldahl's method and is the "humin" nitrogen.

4. The filtered solution is concentrated to 100 c.c. and cooled to 20° C.; 5 grams of sulphuric acid and then 30 c.c. of a solution containing 20 grams of phosphotungstic acid and 5 grams of sulphuric acid per 100 c.c. are added.

5. The precipitate is filtered off after twenty-four hours and washed with a solution containing 2·5 grams of phosphotungstic acid and 5 grams of sulphuric acid per 100 c.c. The washing is effected by rinsing the precipitate from the filter into a beaker and returning to the paper three successive times, each portion of the wash solution being allowed to run out completely before the next is applied. About 200 c.c. of washings are generally obtained.

6. The precipitate is transferred to a 600 c.c. Jena glass flask and the nitrogen estimated in it by Kjeldahl's method, digesting it with 35 c.c. of concentrated sulphuric acid for seven or eight hours. Potassium permanganate crystals may be added three or four times. If the phosphotungstic acid precipitate be small, less sulphuric acid may be used, but sufficient must be taken to prevent bumping.

7. The remaining nitrogen, belonging to the monoamino acids, is found by subtracting the sum of the nitrogen found in the preceding operations from the total nitrogen contained in the protein.

The data given in the table on page 66 show that there are considerable differences in the amounts of the various kinds of nitrogen in proteins.

Protein.	Amide N.	Humin N.	Diamino N.	Monamino N.	Total N.	
Hæmoglobin	1·07	...	0·72 (haematin) 4·07	10·95	16·81	Hausmann
Cryst. Egg Albumin	1·34	0·29	3·30	10·58	15·51	Osborne and Harris
„	1·28	...	3·20	10·17	14·65	Hausmann
Conalbumin	1·21	0·26	4·16	10·49	16·11	Osborne and Harris
Cryst. Serum Albumin	0·95	0·15	4·86	8·81	14·60	Gümbel
Serum Globulin	1·41	...	3·95	10·81	16·17	Hausmann
Edestin (Hemp Seed)	1·88	0·12	5·91	10·78	18·64	Osborne and Harris
„ (Cotton Seed)	1·92	...	5·71	11·01	18·64	„
„ (Sunflower Seed)	2·57	0·24	4·27	11·52	18·58	„
Cryst. Globulin (Squash Seed)	1·28	0·22	5·97	11·04	18·51	„
Globulin (Flax Seed)	2·00	0·22	4·77	11·47	18·48	„
Excelsin	1·48	0·17	5·76	10·97	18·30	„
Legumin (Pea)	1·66 1·72	0·27 0·20	5·24 5·43	10·74 10·56	17·91	„
„ (Vetch)	1·75	0·18	5·17	10·90	18·00	„
„ (Horse Bean)	1·62	0·11	4·92	11·34	17·99	„
„ (Lentil)	1·69	0·11	5·16	11·03	17·99	„
Phaseolin (Kidney Bean)	1·74	0·29	3·97	10·18	16·20	„
Glycinin (Soy Bean)	2·11	0·12	3·95	11·27	17·45	„
Vignin (Cow-Pea)	1·91	0·25	4·28	10·81	17·25	„
Conglutin (Lupine)	2·12 2·65	0·18 0·14	5·20 5·13	10·38 10·30	17·90 18·21	„
Vicilin (Pea)	1·67	0·26	5·12	10·00	17·05	„
Amandin (Almond)	3·05	0·17	4·15	11·55	19·00	„
Corylin (Hazelnut)	2·20	0·16	5·75	10·70	19·00	„
„ (Walnut)	1·78	0·15	5·41	11·51	18·84	„
Globulin (Cocoanut)	1·36	0·14	6·06	10·92	18·48	„
Legumelin (Pea)	1·04	0·38	3·71	10·96	16·09	„
Leucosin (Wheat)	1·16	0·43	3·50	11·83	16·93	„
Glutenin (Wheat)	3·30	0·19	2·05	11·95	17·49	„
Gliadin (Wheat)	4·34	0·07	1·00	12·25	17·66	„
Gliadin (Rye)	4·15	0·11	0·87	12·59	17·72	„
Hordein (Barley)	4·01	0·23	0·77	12·04	17·21	„
Zein (Maize)	2·97	0·16	0·49	12·51	16·13	„
Gliadin (Oat)	3·55	0·26	1·04	10·85	15·70	„
Caseinogen	2·10	...	1·84	11·93	15·87	Hausmann
Caseinogen	1·61	0·21	3·49	10·31	15·62	Osborne and Harris
Vitellin	1·25	0·22	4·65	10·16	16·28	„
Vitellin	0·75	0·25	3·77	10·56	15·33	Plimmer
Livetin	0·68	0·26	3·12	10·90	14·96	„
Gelatin	0·29	...	6·45	11·26	18·00	Hausmann
Keratin (Horn)	1·17	0·42	2·95	11·81	...	Gümbel
Silk-fibroin	0·56	...	...	...	...	Wetzel
Silk-gelatin	8·24	...	10·00	...	...	„
Conchiolin	3·47	...	8·66	...	...	„
Chicken Muscle	1·20	0·44	4·82	9·63	16·09	Osborne and Heyl
Fish Muscle	1·10	0·39	4·95	9·96	16·40	„
Scallop Muscle	1·08	0·40	4·52	11·05	17·05	Osborne and Jones
Ox Muscle	0·89	0·43	4·42	10·44	16·18	„
Protoalbumose A	0·25	...	5·24	11·24	16·80	Pick
Protoalbumose	1·26	...	4·49	12·32	18·07	Friedmann
Heteroalbumose	1·16	...	7·00	10·32	18·48	Pick
Heteroalbumose	0·36	...	6·27	10·03	16·89	Friedmann

B. Distribution of the Nitrogen in Seven Groups.

Further differentiation of the units composing the protein molecule into those containing amino groups and those containing nitrogen in heterocyclic combination was only possible after the discovery of proline and tryptophane in 1901 and of oxyproline in 1902.

This subdivision is possible, since nitrous acid reacts only with amino groups with liberation of nitrogen :—

$$\begin{matrix} CH.NH_2 \\ | \\ COOH \end{matrix} + HNO_2 = \begin{matrix} CHOH \\ | \\ COOH \end{matrix} + N_2$$

the amount of nitrogen evolved being double that contained in the amino acid.

The action of nitrous acid upon amino acids and amides as a method for estimating these compounds was introduced by Sachsse and Kormann in 1875. These investigators employed an apparatus consisting of a small cylinder, furnished with a rubber stopper, through which two tap funnels and an exit tube for the evolved gas passed. Potassium nitrite was placed in the cylinder, dilute sulphuric acid in one of the tap funnels and the solution of the substance (0·6-1·0 gram) in the other. The exit tube was placed under an eudiometer filled with ferrous sulphate solution to absorb the nitric oxide. Air was expelled from the apparatus by the decomposition of some of the nitrite with the acid. As soon as the expulsion was complete, the amide solution and more acid solution were allowed to enter into the cylinder and the gas collected. The ferrous sulphate solution freed the mixture from nitric oxide and more was added, if necessary. Carbon dioxide was then removed with potash and the remaining gas was measured.

This method was exhaustively tested in the Guinness Research laboratory by Horace Brown and J. H. Millar, who published their experiences in 1903. They found that there were several serious sources of error in the original method before they could apply it to their own subject of investigation. These were due (1) to residual air contained in the apparatus, or in the liquids; (2) to the difficulty of absorbing the excess of nitric oxide with ferrous sulphate. Carbon dioxide was used to remove the air and the evolved nitrogen, and difficulty was experienced in obtaining a steady and constant stream of this gas. Pure carbon dioxide was prepared by the action of 30 per cent. sulphuric acid on normal sodium carbonate solution. A constant evolution of gas was not obtained, when the two solutions were allowed to drop separately into a flask, owing to the supersaturation of

the liquid with the gas. A steady stream of carbon dioxide was obtained by allowing the liquids to mix in a piece of wide glass tubing, which was drawn out at its lower extremity so that only a small aperture remained and closed at its upper end by a small cork through which tubes connected with reservoirs of acid and carbonate passed; two small apertures in its sides allowed for the escape of the gas. This wide tube was placed in the vessel used for the generation of the carbon dioxide. The mixture of acid and carbonate solutions accumulated to a height of about 3 cm. in the tube, and then dropped into the flask, which could be emptied by an attached syphon tube. The carbon dioxide entered the apparatus through a special trap. Air contained in the apparatus was removed by a stream of carbon dioxide and steam, which was made to enter through this trap. All the solutions used were made with air-free water, charged with carbon dioxide; the same water was used for washing purposes.

The carbon dioxide was removed by the potash solution contained in a Lunge nitrometer, with which the exit tube of the apparatus was connected. The excess of nitric oxide was removed by means of oxygen, prepared by the electrolytic decomposition of water, and passed into the nitrometer, and the excess of oxygen by passing the gas in the nitrometer into a double Hempel pipette containing pyrogallol dissolved in 60 per cent. potash solution.

Allowing one to two hours for the reaction of the nitrous acid upon the amino acids, satisfactory results were obtained with glycine, alanine, phenylalanine, leucine, aspartic acid, glutamic acid and asparagine. The results with tyrosine were not satisfactory, but they found that it reacted quantitatively after bromination.

For the application of this method to the differentiation of the various forms of nitrogen in proteins we are indebted to Van Slyke, who not only has devised a much simpler apparatus for the estimation of the amino-nitrogen, but also has devised a means for carrying out the estimation of the two groups of monoamino acids, the several diamino acids and cystine in one series of operations.

I. Estimation of Amino Nitrogen.

The estimation of the amino nitrogen is performed in an apparatus very similar to that used by Sachsse and Kormann and by Horace Brown and Millar; the apparatus is filled with nitric oxide, which gas is also used for washing the evolved nitrogen into the eudiometer; excess of nitric oxide is removed with permanganate contained in a Hempel pipette. Fig. 2 shows the apparatus designed by Van Slyke.

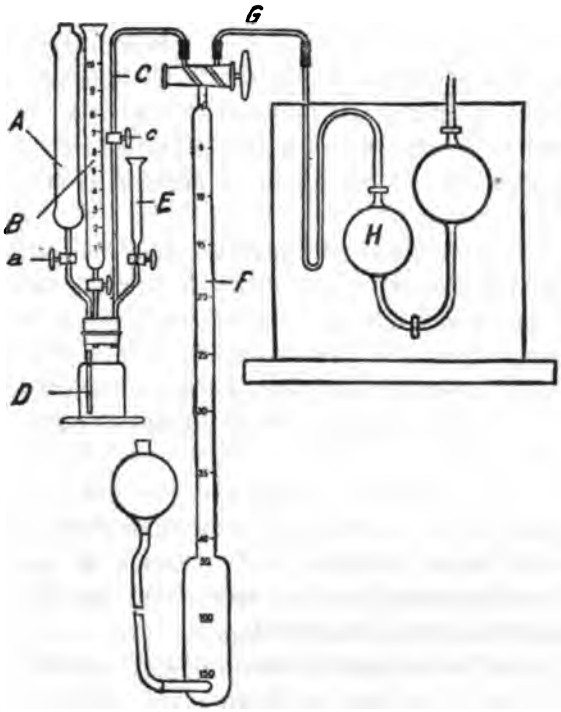


FIG. 2.

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It consists of a vessel D of 35-37 c.c. capacity for the interaction of the nitrous acid and the solution of the amino substance. Connected with this vessel, which is fitted with a four-holed rubber stopper held in place by a wire, or by an external screw stopper, are

A, a vessel of 35 c.c. capacity to hold water for displacing air from D, or receiving solution from D.

B, a 10 c.c. burette containing the solution of the amino substance.

C, the exit tube for the evolved gases; its lower end is flush with the stopper. It is connected with the gas burette F.

E, a small cylinder for the introduction of amyl alcohol to solutions containing proteoses, or proteins, which froth considerably when treated with nitrous acid. An occasional drop during the evolution of the nitrogen generally suffices.

The connecting tubes are of 6-7 mm. external diameter and 1 mm. bore; the tube to A is of 2 mm. bore.

The gas burette F is of 150 c.c. capacity; its upper and narrower portion holding 40 c.c. is graduated in tenths; its lower and wider portion in 10 c.c. divisions. This large burette will hold all the gas which is liberated whilst the apparatus is freed from air. The gas, which is to be finally measured, should only fill a portion of the narrow accurately graduated part. It is filled with 1 per cent. sulphuric acid. G connects the gas burette with the Hempel pipette H, which contains a solution of 50 grams of potassium permanganate and 25 grams of caustic potash per litre.

The nitric oxide reduces the permanganate, but the manganese dioxide formed is in such a fine state of division that it does not interfere with the manipulation. Several determinations can be made with the same solution. Deposition of manganese dioxide in the capillary tube G is prevented by allowing water from the gas burette to remain in this tube, instead of permanganate. Any carbon dioxide evolved is absorbed by the alkali.

Excess of sodium nitrite solution (30 gm. per 100 c.c.) is used and it is decomposed by glacial acetic acid; less nitric oxide is evolved by its use than by the use of mineral acid. The amino substance may be dissolved in semi-normal acid, 50 per cent. acetic acid, or normal alkali.

Only a small correction for the reagents is necessary; commercial nitrite gave 0·2 c.c. of nitrogen for five minutes' reaction, 0·3 c.c. for thirty minutes and 0·5 c.c. for two hours.

The estimation is performed in the following way:—

1. The amino solution, containing less than 20 milligrams of amino nitrogen, is placed in B and 5 c.c. of water in A; 28 c.c. of the nitrite solution is placed in D and then 7 c.c. of glacial acetic acid. Whilst the nitric oxide is being evolved, the vessel D is closed with the stopper, the stop-cock *c* in the exit tube C being open. The small volume of air in D is driven out by letting water enter from A till the bottle is filled and liquid rises in C.

The air dissolved in the nitrous acid solution is removed by closing *c*, leaving *a* open and shaking D. It is best to hold A, B, C in the left hand during this operation. Rapid evolution of nitric oxide occurs

which gathers in D and forces 10-15 c.c. of solution back into A. The cock *c* is now again opened and the nitric oxide, together with the air swept out of the solution, is forced out of D by liquid from A. This process is repeated to ensure removal of all the air.

By closing *c* and shaking D a gas space of about 20 c.c. is made, so as to make room for the amino solution from B.

G and H are filled with permanganate; F is filled with the sulphuric acid solution up to the top of the rubber piece connecting it to C.

Tap *a* is closed and tap *c* is opened. These manipulations require about two minutes.

The solution from B is run into D and mixed with the nitrous acid. The evolution of nitrogen commences immediately; after five minutes D is thoroughly shaken to complete the evolution of the gas.

If substances other than *α*-amino acids are being estimated a drop of amyl alcohol from E is occasionally added and the solution shaken several times per minute. Some compounds require longer than five minutes for complete decomposition; in this case the reaction is continued for a period of fifteen minutes to two hours.

When the reaction is completed the gas from D and C is driven into F by opening *a* and letting liquid run from A into D.

The gas is then driven from F into H by raising the levelling bulb. No gas must be left in the capillaries of G and the pipette. By shaking the gases with the permanganate the nitric oxide is absorbed. The residual nitrogen is run back into F, the permanganate filling G as far as the tap of F. The volume of gas in F is then measured by bringing the surface of the liquid in the bulb even with the meniscus. Generally, one shaking with the fresh permanganate suffices to remove all the nitric oxide, but it is advisable to test if the absorption is complete by returning the gas to the Hempel pipette and again measuring. The weight of nitrogen corresponding to the volume of gas is calculated in the usual way. The results are divided by 2 (see equation, p. 67).

Each milligram of amino substance gives off from 1·7 to 1·9 c.c. of nitrogen.

A correction should be made for the air—0·2 c.c.—dissolved in 10 c.c. amino solution, *i.e.*, allowing for the oxygen which combines with the nitric oxide forming peroxide and is absorbed by the permanganate, 0·16 c.c. must be deducted from the volume of gas. This correction is equivalent to 0·09 milligrams of amino nitrogen. No correction is necessary, if air-free water be used in preparing the amino solution.

Except lysine, the natural amino acids all react quantitatively in five minutes: half an hour is required for this substance. Ammonia and methylamine require one and a half to two hours, purines and pyrimidines two to five hours, and urea eight hours.

The completeness of the reaction may be tested by repeating the process and ascertaining if more gas is evolved.

Glycine and cystine evolve a larger volume of gas than the theoretical; the factor ·926 may be used in estimating cystine; 3 per cent. of the total volume of gas must be deducted for glycine.

Klein has since constructed a simpler and more permanent apparatus for this estimation; it is reproduced in fig. 3.

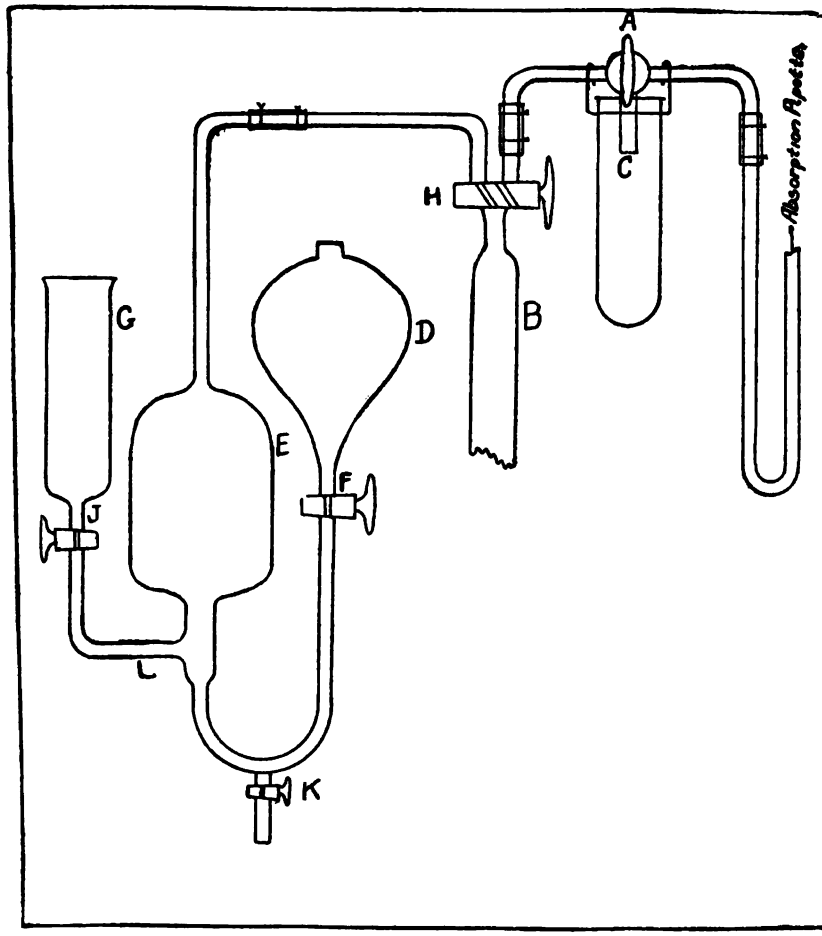

FIG. 3.

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All the glass tubing is of medium bore capillary tubing.

The manipulation is as follows:—

Draw the liquid from the absorption pipette through the three-way stop-cock A, which is then turned so that it connects with the burette B and with the air at C.

Pour 28 c.c. nitrate solution into reservoir D; draw it into E by lowering the levelling bulb of the burette B, leaving a little liquid above the cock F.

Discharge the air in the burette B through A; it is not necessary to fill the capillary tube with liquid.

Pour the solution to be analysed into G.

Then pour 7 c.c. of glacial acetic acid into D and allow it to flow into E by lowering the levelling bulb of the burette. Some acid must remain above the stop-cock F.

The gas evolved displaces the air in E. When 45-50 c.c. of gas have been collected in the burette, close H and open F. A gas space of sufficient size soon forms in E.

Meanwhile drive the gas out of the burette through A, filling the capillary with liquid and allowing the excess to run into the test tube C.

Close H and turn A so as to connect the absorption pipette with B.

Close F and draw liquid from G into E, being careful not to run it below J.

Close J; wash G with a little water and run it into E. Repeat two or three times.

When the reaction has proceeded for five minutes, draw the liquid from D into E until it reaches H.

Force the gas from the burette into the absorption pipette so that the acid solution fills the capillary of the pipette. The time of absorption of the nitric oxide is reduced, if the pipette be shaken whilst the gas is passed into it.

The unabsorbed gas is returned to the burette, the permanganate being drawn as far as H.

The gas is then measured; further treatment with permanganate and further time for the reaction may be given by repetition of the processes.

The apparatus is cleaned by forcing out the liquid through K. Distilled water is admitted through D and G into E and the vessels washed two or three times. It is convenient to support the apparatus on a grooved cork at L.

Amyl alcohol, if necessary, is added through D before the admission of the nitrite solution, or through G.

II. Estimation of the Different Groups of Amino Acids.

The estimation of the several groups of amino acids present in a protein is effected by the following series of operations :—

1. *Hydrolysis.*—3 grammes of protein, or better 6 grammes for duplicate analyses, are dissolved in 10 or 20 parts of 20 per cent. hydrochloric acid and boiled in a tared flask under a reflux condenser.

After six or eight hours the hydrolysis is stopped. Portions of 1 c.c. or 2 c.c. (enough to contain 0·1 gramme protein) are withdrawn with a pipette and diluted to 10 c.c. In these portions the amount of amino nitrogen is determined, the reaction being allowed to proceed for five minutes standing and then for one minute with shaking. Under these conditions the same proportion of ammonia (15-20 per cent.) is decomposed in each determination.

The hydrolysis flask is weighed and the hydrolysis continued for another period of six or eight hours, when amino nitrogen is again determined.

Hydrolysis is continued until the amino nitrogen is constant.

The object of weighing is to ascertain if the solution has become concentrated by loss of vapour and to allow of a correction for a decrease of the volume.

2. *Estimation of Total Nitrogen.*—The products of hydrolysis are transferred to a measuring flask of 100 c.c or 250 c.c capacity. Total nitrogen is estimated by Kjeldahl's method in an aliquot portion containing 0·2 gramme of protein. All the subsequent estimations are based upon this value.

3. *Amide Nitrogen.*—Since cystine is very easily decomposed by boiling with magnesia at 100° the determination of ammonia must be carried out *in vacuo* at 40°, or at room temperature by the aeration method of Denis (*J. Biol. Chem.*, 8, 427). The method of distilling *in vacuo* is to be preferred as the same apparatus is repeatedly employed in the other estimations.

The distillation *in vacuo* is performed in the apparatus shown in fig. 4. (p. 75).

The Claisen flask and receiver are of 1 litre capacity, the guard flask of 200 c.c.

The hydrolysed solution is placed in the double-necked flask and diluted to 200 c.c; 100 c.c of alcohol are added to prevent frothing and then an excess of a 10 per cent. suspension of calcium hydrate, as

shown by the turbidity and alkaline reaction of the solution. Air may be introduced through the stop-cock, if distillation starts too rapidly; the stop-cock serves to release the vacuum when the distillation is finished. The decinormal acid in both flasks is mixed and titrated with decinormal soda using alizarin sulphonate as indicator, which may be added previous to the distillation.

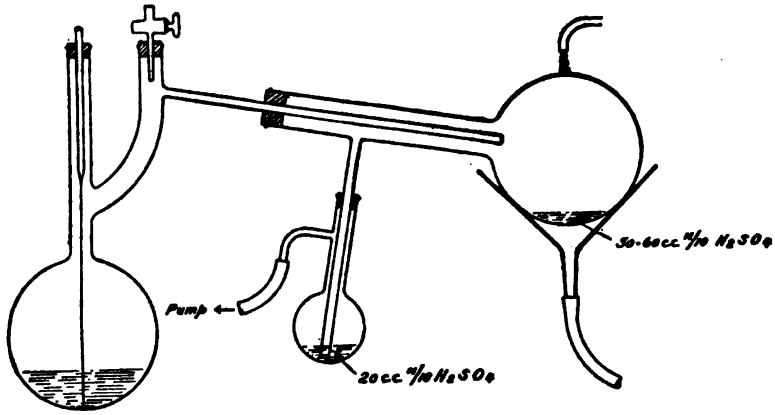

FIG. 4.

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4. *Humin Nitrogen.*—The black colouring matter is adsorbed by the lime, which is filtered off on a folded filter paper and washed with water until the washings are free from chlorides. The precipitate and paper are then submitted to Kjeldahl analysis, using 35 c.c. of sulphuric acid.

5. *Precipitation and Washing of the Phosphotungstate Precipitate.*—The filtrate from the humin nitrogen is neutralised with hydrochloric acid, returned to the vacuum distillation apparatus and concentrated in to about 100 c.c.

It is then washed into a 250 c.c. conical flask, and 18 c.c. of concentrated hydrochloric acid and 15 grammes of phosphotungstic acid[1] in aqueous solution are added.

The entire solution is diluted to 200 c.c. with water and heated in a water-bath, until the precipitate has nearly, or quite, redissolved. On cooling, the phosphotungstates separate in a crystalline form.

After standing for at least forty-eight hours they are filtered off and washed in the following manner:—

[1] This should be purified by Winterstein's method by shaking its acid solution with ether; from the ethereal layer containing the substance the acid is recovered by recrystallisation.

A 3-inch Buchner funnel is covered with a hardened filter paper of such a size that it fits against the bottom and side walls; the portion of the paper against the side walls is folded into about twenty plaits so that it fits snugly all round.

The precipitate is poured into this pocket and the mother liquor removed by suction and by pressing down the precipitate with a flattened rod.

The filtrate is returned to a beaker.

Washing is effected with 10-12 c.c. of a solution containing 2·5 grammes of phosphotungstic acid and 3·5 grammes of hydrochloric acid per 100 c.c.; this is first used to dislodge the particles remaining in the flask; it is then poured upon the precipitate which is stirred up until all lumps are broken and until there is only a granular suspension. It is then sucked dry as before. The washing is repeated three to four times in this manner. Then the precipitate on the filter is washed five to ten times with the same solution from a wash bottle, commencing round the edges and sucking dry each time.

It frequently happens that the later washings run through somewhat turbid; these are filtered through a folded paper. All washings are combined with the main filtrate. The washing must be continued till the liquid is free from calcium; 1 c.c. of the filtrate when tested with oxalic acid in 3 per cent. sodium hydrate must give no turbidity even after standing for several minutes.

6. *Treatment of the Phosphotungstate Precipitate.*—The precipitate is removed with a spatula, as completely as possible, to a beaker of over 1 litre capacity. The filter paper is then spread out on the bottom of a basin and washed with water made just alkaline with a few drops of 20 per cent. sodium hydrate. The small folded paper is treated in the same way. The particles of precipitate are dissolved by the soda, and any granules remaining in the original flask are dissolved in the same way. The alkaline solutions and washings are poured into the beaker containing the main bulk of the precipitate. The whole is then carefully dissolved in soda by adding 50 per cent. alkali, drop by drop, with continual stirring. Phenolphthalein is added as indicator; as soon as the solution becomes red, addition of the alkali must be stopped; if the colour disappears, more alkali, but only to the excess of three or four drops, must be added. A red solution must finally result.

The solution is diluted to 800 c.c. and the phosphotungstic acid is removed by slowly adding, in portions of a few c.c., a 20 per cent. solution of barium chloride, until a test portion gives an immediate

granular precipitate with neutral sodium sulphate solution. If the red colour disappears in the process two or three more drops of alkali are added. A large excess of barium chloride should be avoided.

The barium phosphotungstate is filtered off using the same funnel, paper and precautions as before, except that larger portions of wash water may be used. The final washing must give no reaction for chlorides.

The filtrate and washings are concentrated *in vacuo*, until they are reduced in volume to 50 c.c. The barium phosphotungstate, which separates out, is filtered off, and the filtrate and washings received in a 200 c.c. double-necked distilling flask; the volume is then again reduced and made up to 50 c.c. in a measuring flask.

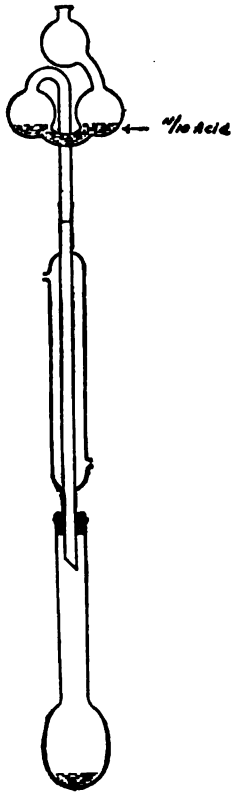

FIG. 5.
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7. Estimation of Arginine.—Since arginine is quantitatively decomposed by boiling with alkali with the loss of half of its nitrogen, its estimation is performed before that of the total nitrogen contained in the solution.

25 c.c. of the solution are placed in the 200 c.c. flask of the apparatus shown in fig. 5.

The Folin bulbs are connected to the flask either by a ground glass joint, or by a heavy piece of rubber tubing. These bulbs contain 15 c.c. of decinormal acid, coloured with alizarin sulphonate. 12·5 grams of solid potash and a piece of porous porcelain are added to the solution in the flask, and the solution is boiled gently for exactly six hours. Nearly all the evolved ammonia diffuses into the bulbs.

The bulbs are disconnected and 100 c.c. of water are poured through the condenser into the flask. The flask is connected with the condenser of a Kjeldahl distilling apparatus, and the remainder of the ammonia driven off and collected in the acid from the bulbs, which has been transferred to a suitable receiver. Not more than 100 c.c. of the solution must be distilled over as the strong potash may destroy the other substances in the solution.

The excess of acid in the receiver is titrated. Each c.c. of acid neutralised by the ammonia corresponds to 0·0028 gram arginine-nitrogen, or 0·0056 gram in the total solution.

Cystine is usually not present in sufficient amount in proteins to cause any considerable error in this determination, but if a keratin be under investigation a correction must be made; 18 per cent. of its nitrogen is evolved when it is boiled with alkali; the nitrogen figure of cystine is obtained later in the process.

8. *Total Nitrogen of the Hexone Bases and Cystine.*—The solution remaining from the arginine determination is transferred to a Kjeldahl flask of 500 c.c.; 35 c.c. of sulphuric acid are added and 0·25 gram of copper sulphate. The nitrogen is then estimated in the usual way.

The sum of the No. of c.c. decinormal acid neutralised + the No. of c.c. of decinormal acid previously neutralised in the arginine determination, multiplied by 0·0028, gives the total nitrogen content.

9. *Estimation of Cystine.*—Cystine is estimated by determining the total sulphur content by Denis' modification of Benedict's method. 10 c.c. of the solution of hexone bases + cystine from operation (6) are placed in a porcelain dish of 7 to 10 cm. diameter with 5 c.c. of a solution containing 25 grams copper nitrate, 25 grams sodium chloride and 10 grams ammonium nitrate per 100 c.c. The mixture is evaporated to dryness on the water-bath and then gradually heated to redness, at which temperature it is maintained for ten minutes. The residue is dissolved in 10 c.c. of 10 per cent. hydrochloric acid and diluted to 150 c.c. 10 c.c. of a 5 per cent. solution of barium chloride are slowly added to the boiling solution and the barium sulphate is filtered off and washed in the usual way. A correction must be made for the amount of sulphate in the reagents which should not exceed 1·5 milligrams barium sulphate.

1 milligram $BaSO_4$ corresponds to 0·06 milligram cystine nitrogen in the 10 c.c. analysed, *i.e.*, to 0·3 milligram cystine nitrogen in the total solution of bases + cystine.

10. *Estimation of Amino Nitrogen.*—10 c.c. of the solution are used for this estimation (p. 69). The reaction must be allowed to proceed for half an hour at 20° C., or longer at a lower temperature; similarly for the blank determination for the reagents.

A correction must be made for cystine which gives 107 per cent. gas; as before it applies only to those proteins—keratins—which contain a large amount of cystine.

Calculation of Histidine Value.

Total nitrogen of bases minus total amino nitrogen gives the total non-amino nitrogen, D. This is contained in the arginine and histidine.

Since three-fourths of the arginine nitrogen and two-thirds of the histidine nitrogen are in this form, the total non-amino nitrogen minus three-fourths of the arginine nitrogen (found previously) represents two-thirds of the histidine nitrogen. Hence

$$\text{Histidine N} = \tfrac{3}{2}(\text{D} - \tfrac{3}{4}\,\text{Arg.})$$
$$= 1{\cdot}667\ \text{D} - 1{\cdot}125\ \text{Arg.}$$

Calculation of Lysine Value.

Knowing the other values, that of lysine is found by difference:—

$$\text{Lysine N} = \text{Total N} - (\text{Arg. N} + \text{Cyst. N} + \text{Hist. N}).$$

11. *Determination of the Total Nitrogen of the Monoamino Acids.*— To the combined filtrate and washings from the phosphotungstate precipitate 50 per cent. caustic soda is carefully added, until the solution becomes turbid by precipitation of lime and is just alkaline. Excess must be carefully avoided as the precipitate may then not completely dissolve in acetic acid, which is added to clear the solution when just alkaline. The acid solution is concentrated *in vacuo* until salt commences to crystallise out. The solution is then washed into a 150 c.c. measuring flask and diluted up to the mark.

Total nitrogen is estimated in 25 c.c. portions by Kjeldahl's method, using 35 c.c. of sulphuric acid, 15 grams potassium sulphate and 0·25 grams of copper sulphate. The acid must be added carefully on account of the evolution of hydrochloric acid. The digestion must be continued for three hours after the solution has become clear.

12. *Determination of the Amino Nitrogen of the Monoamino Acids.*— 10 c.c. portions of the solution are used for this purpose and the time for the reaction with nitrous acid is six to ten minutes.

The volume of nitrogen evolved by a given amount of amino nitrogen is 2·5 times the volume of decinormal acid neutralised, if the same amount is determined by the Kjeldahl method. Therefore, the portions of 25 c.c. and 10 c.c. used in these last estimations give results of similar accuracy.

13. *Corrections for the Solubilities of the Phosphotungstates.*— Although the conditions of precipitation are not always the same, the variation is not sufficient to cause significant change in the solubilities of the phosphotungstates. When precipitated from a volume of 200 c.c. the following corrections should be added, and the sum of the figures for amino nitrogen and non-amino nitrogen subtracted from the figures for the monamino acids :—

	Total N. Gram.	Amino N. Gram.	Non-Amino N. Gram.
Arginine N. . . .	0·0032	0·0008	0·0024
Histidine N. . . .	0·0038	0·0013	0·0025
Lysine N. . . .	0·0005	0·0005	0·0000
Cystine N. . . .	0·0026	0·0026	0·0000
Sum		0·0052	0·0049

This long method has been rigidly tested by Van Slyke upon pure amino acids singly, and when mixed together, and upon typical proteins for which he gives the following values expressed in percentages of the total nitrogen :—

	Amide N.	Humin N.	Cystine N.	Arginine N.	Histidine N.	Lysine N.	Monoamino N.	Non-Amino N.	Total N.
Gliadin . . (Wheat)	25·52	0·86	1·25	5·71	5·20	0·75	51·98	8·50	99·77
Edestin . .	9·99	1·98	1·49	27·05	5·75	3·86	47·55	1·70	99·37
Keratin . . (Dog's Hair)	10·05	7·42	6·60	15·33	3·48	5·37	47·50	3·10	98·85
Gelatin . .	2·25	0·07	0·00	14·70	4·48	6·32	56·30	14·90	99·02
Fibrin . .	8·32	3·17	0·99	13·86	4·83	11·51	54·30	2·70	99·58
Hæmocyanin .	5·95	1·65	0·80	15·73	13·23	8·49	51·30	3·80	100·95
Hæmoglobin .	5·24	3·60	0·00	7·70	12·70	10·90	57·00	2·90	100·04

The accuracy of the method is borne out by the figures under the column of total nitrogen; except in one case, the nitrogen recovered is within 1 per cent. In the duplicate analyses of these proteins the maximum differences were 2·14 per cent. for the histidine (edestin) and 1·60 per cent. for the monoamino nitrogen (hair). These differences were more than twice any other deviations from the figures in the series. The highest difference was 0·79 per cent. for histidine and the lowest 0·05 for cystine.

The correspondence between these figures and the actual amounts of amino acid isolated from the protein is fairly good. The high proline content of gelatin was well known, but the non-amino nitrogen content of 15 per cent. of the total nitrogen of this protein is very striking. Further, the large amount of lysine in hæmoglobin was unexpected; hæmoglobin has always been supposed to be composed chiefly of histidine, when the diamino acids were considered.

Further data will be awaited with interest so that better comparisons can be made. The present data will no doubt stimulate the chief workers on the hydrolysis of the proteins to obtain better yields of certain of the units.

SECTION II.

THE CHEMICAL CONSTITUTION OF THE UNITS, OR THE DISCOVERY AND SYNTHESES OF THE AMINO ACIDS.

IN Section I. an account was given of how the units of the protein molecule are now isolated and estimated, and the results were embodied in several tables. In general, it may be said, that the amino acids were first discovered in that protein in which they occurred in the largest amounts. An account will be given in this section of the discovery and of the determination of the constitution of each amino acid.

A. MONOAMINOMONOCARBOXYLIC ACIDS.

Glycine.

This, the simplest of the products of hydrolysis of the proteins, was also the first to be discovered; it was obtained by Braconnot, in 1820, by boiling gelatin with dilute sulphuric acid, and on account of its sweet taste he called it sugar of gelatin. In 1846 Dessaignes obtained it from hippuric acid by hydrolysis, and, in 1848, Strecker showed that cholic acid (now glycocholic acid) consisted of this amino acid and cholalic acid, so that, as a constituent of substances of animal origin, it became of great importance. Its presence in elastin was demonstrated by Jeanneret, in horn by Horbaczewski, in spongin by Krukenberg, in conchiolin by Wetzel, and in silk-fibroin by Cramer; Faust and Spiro showed that it was present in globulin. It does not occur in albumin, nor in caseinogen, nor in hæmoglobin; it is present only to a small extent in the vegetable proteins, and for this reason it was not isolated until Abderhalden showed its presence in these proteins by Fischer's ester method.

In the free state, glycine was found by Chittenden in an extract of the American mussel, *Pecten irradians*, and of recent years it has been recorded as sometimes occurring in the urine.

Its elementary composition of $C_2H_5NO_2$ was first correctly deter-

mined in 1846 by Mulder and by Laurent, and in that year, after Dessaignes had pointed out the unsuitability of the name given to it of sugar of gelatin, as there were other substances like it with a sweet taste and which were not fermentable, its name of glycocoll (γλυκύς, sweet, κόλλα, glue) originated with, and was first used by, Horsford, who made an extensive study of it and its derivatives, whilst working in Liebig's laboratory where much of the early work on proteins was carried out.

Laurent regarded glycocoll as belonging to the ammonia type of organic compounds; it was supposed by Cahours to be a derivative of acetic acid, which supposition was only proved by its synthesis from bromacetic acid and ammonia by Perkin and Duppa, and from chloracetic acid and ammonia by Cahours, both in 1858:—

$$CH_2Cl.COOH + NH_3 = HCl + CH_2.NH_2.COOH.$$

About this time the terms glycocine and glycine were used for glycocoll, as this was then recognised as a homologue of alanine and leucine. The whole of this series of compounds was named the *glycines*.

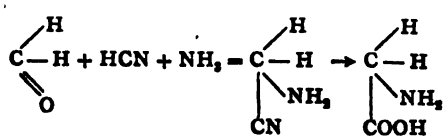
A very interesting synthesis of glycine was described by Emmerling, in 1873, by the action of hydriodic acid upon cyanogen; here the hydriodic acid acts both as a reducing agent and as a hydrolysing agent:—

$$\begin{matrix}CN\\|\\CN\end{matrix} + 5HI + 2H_2O = \begin{matrix}CH_2.NH_2\\|\\COOH\end{matrix} + NH_4I + 2I_2,$$

and, in 1877, Wallach obtained it by the reduction of cyanoformic ester with zinc:—

$$\begin{matrix}COOC_2H_5\\|\\CN\end{matrix} + 4H = \begin{matrix}COOC_2H_5\\|\\CH_2.NH_2\end{matrix}.$$

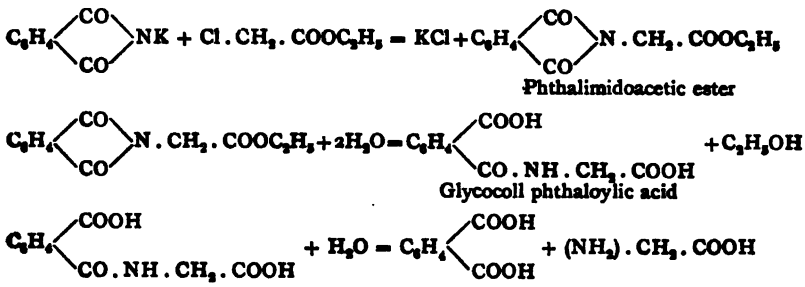
Lubavin, in 1882, stated that glycine was formed by the action of ammonium cyanide upon glyoxal, which probably first breaks down into formaldehyde and then, by the cyanhydrin reaction, yields glycine:—



The direct synthesis of glycine from formaldehyde was only carried out in 1894 by Eschweiler. This method, as well as the method from chloracetic acid and ammonia, by which Nencki and also Mauthner and Suida, by slight modifications in technique, attempted to obtain

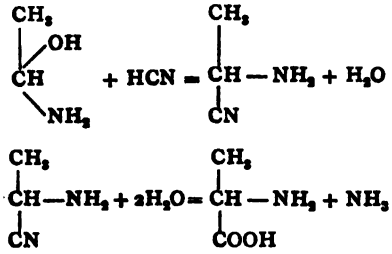
larger yields, only give about 20 per cent., but the method described by Gabriel and Kroseberg, in 1889, who made use of Gabriel's phthalimide reaction, gives an almost theoretical yield of glycine, as was first shown by Goedeckemayer; this reaction takes place in the following stages:—

Phthalimidoacetic ester is obtained by the action of chloracetic ester upon potassium phthalimide; this is first hydrolysed by alkali to glycocoll phthaloylic acid, and then by 20 per cent. hydrochloric acid to glycocoll and phthalic acid:—



Alanine.

Of the naturally occurring amino acids alanine alone was prepared synthetically, many years before it was discovered as a constituent of the protein molecule. Its name was given to it by its discoverer, Strecker, who prepared it in 1850 from aldehyde ammonia, which, when treated with hydrogen cyanide gives the aminocyanohydrin, and this, by hydrolysis, is then converted into the amino acid :—



Owing to the ease with which the aldehyde resinifies in presence of alkali and potassium cyanide the yield of alanine is very poor. If, however, the reaction be carried out in the presence of excess of ammonium chloride and if the potassium cyanide be slowly added to the aldehyde dissolved in ether, a yield of alanine amounting to 60 to 70 per cent. can be obtained, as has been recently shown by Zelinsky and Stadnikoff.

This is the first of the general methods employed in the synthesis of the amino acids.

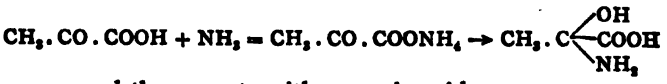
Alanine was prepared in 1860 by Kolbe by the second general method, i.e. by the action of ammonia upon *a*-chloropropionic acid :—

$$CH_3.CHCl.COOH + NH_3 = HCl + CH_3.CH(NH_2).COOH,$$

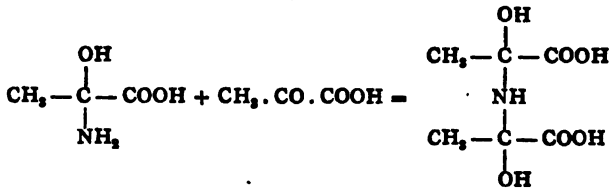
and in 1864 by Kekulé from monobromopropionic acid and alcoholic ammonia.

A synthesis of acetylalanine, from which alanine can be obtained by hydrolysis, was described in 1900 by de Jong. Pyruvic acid was neutralised with ammonium carbonate; there was a considerable rise in temperature, carbon dioxide being evolved, and the ammonium salt of acetylalanine crystallised out. The explanation of this reaction is based upon Erlenmeyer and Kunlin's synthesis of phenylalanine from phenylpyruvic acid and it proceeds as follows :—

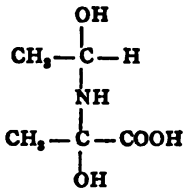
Ammonium pyruvate, which is first formed, is tautomeric with *a*-amino-oxypropionic acid, into which it changes :—



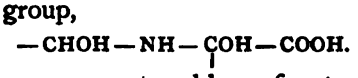
This compound then reacts with pyruvic acid:—



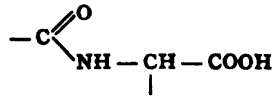
and the compound thus formed loses carbon dioxide giving the compound,



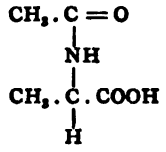
which possesses the group,



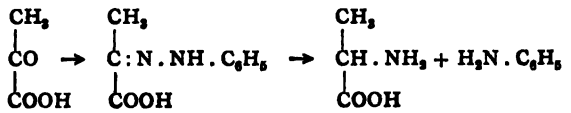
By intramolecular rearrangement and loss of water this becomes



The above compound by rearrangement and loss of water is thus converted into acetylalanine:—



Fischer and Groh, in 1911, showed that some amino acids were most readily prepared by the reduction of the phenylhydrazone of the corresponding ketonic acid with aluminium amalgam. Though it is not the most advantageous method for the preparation of alanine, this compound is easily obtained by the reduction of the phenylhydrazone of pyruvic acid:—



The occurrence of alanine in proteins was first demonstrated by Schützenberger, who did not actually identify his product with the synthetical one; Weyl in 1881 obtained it as a decomposition product of silk and showed that his preparation was similar in properties to Strecker's synthetical alanine. He thus established it as a constituent of a protein molecule. The researches of Emil Fischer have shown that alanine is a constant constituent of all proteins. It is worthy of note that of the eighteen definitely determined units of a protein molecule, six of them, namely, isoleucine, phenylalanine, tyrosine, serine, histidine and tryptophane, are derivatives of *a*-aminopropionic acid.

Valine.

A body of the composition $C_5H_{11}NO_2$ was obtained in 1856 by v. Gorup-Besanez from an aqueous extract of pancreas, and on account of its similarity in properties to leucine he regarded it as a homologue of leucine and termed it butalanine. Schützenberger, in 1879, also obtained a substance which had this empirical formula and properties like that of leucine.

An aminovalerianic acid was described in 1883 by Schulze and Barbieri as occurring in the seedlings of yellow lupines, and subsequently Schulze again isolated it from the extracts of other seedlings. It appeared to correspond to n-aminovalerianic acid, which had been synthesised by Lipp.

In 1899 Kossel isolated a similar substance from the protamine clupeine of herring milt, and since then E. Fischer and his pupils have obtained it from caseinogen, horn and other proteins. The preparation from horn, when racemised, corresponded in properties with the synthetical *a*-aminoisovalerianic acid,

$$\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\rangle CH \cdot CH(NH_2) \cdot COOH,$$

which had been first prepared by Clark and Fittig in 1866 from the corresponding bromo-compound and later by Lipp in 1880 from isobutyraldehyde; its derivatives were identical with those of this acid which were prepared by Slimmer in 1902. The exact identity of the natural and synthetical substances was only established in 1906, when Fischer prepared d-aminoisovalerianic acid from the synthetical product, and showed that its specific rotation was the same as that of Schulze and Barbieri's natural substance. The name valine was given to this compound in 1906 by E. Fischer.

Leucine.

A substance, corresponding to our leucine, was described by Proust in 1818 under the name of oxide-caséux. Two years later, in 1820, Braconnot isolated from the products resulting by boiling meat with dilute sulphuric acid a substance which he named leucine on account of its glistening, white (λευκός) appearance. Mulder, in 1839, obtained it by boiling meat with alkali and by the putrefaction of casein. Its occurrence and oxidation products were investigated by Liebig, who regarded it as one of the constituents of the protein molecule, as was proved in 1849 by Bopp, who prepared it from caseinogen, fibrin and albumin by fusion with potash, and also by hydrolysis with acids and by putrefaction. Hinterberger showed that it was present in horn, and Zollikofer in elastin.

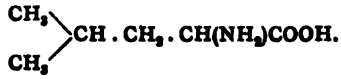
Leucine also occurs in the free state in the various organs of the animal body as has been pointed out by Frerichs and Städeler and many other observers.

Not only is it present in animal, but also in vegetable, proteins, from which it passes by the action of enzymes into the extracts of germinating seedlings, as shown by Schulze and his co-workers. Leucine is, with the exception of arginine, the most widespread of all the units which go to make up the protein molecule.

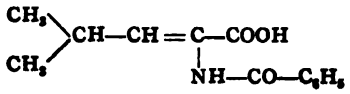
Its correct empirical formula $C_6H_{13}NO_2$ was first given to it by Laurent and Gerhardt. These observers and also Cahours showed that it belonged to the glycine series of compounds; Liebig and others showed that on oxidation it gave ammonia and valerianic acid, and also valeronitrile, and Strecker obtained leucic acid by treating it with nitrous acid. But only in 1868, when Hüfner obtained caproic acid and ammonia by reducing it with hydriodic acid, was it shown to be an *α*-aminocaproic acid. Hüfner tried to prove this by comparing the natural leucine with two synthetical leucines; (1) that prepared by the action of ammonia on bromocaproic acid obtained from the fermentation caproic acid, and (2) that prepared from isovaleraldehyde, hydrogen cyanide and ammonia which had been first synthesised by Limpricht in 1855. Neither of these two synthetical leucines corresponded exactly with natural leucine, and Hüfner regarded them as identical compounds rather than as isomers.

The question of the constitution of leucine was again taken up in 1891 by Schulze and Likiernik. The natural product is optically active, but on heating with baryta at 160° C. it is racemised; this

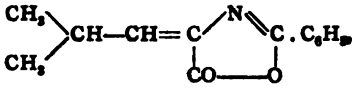
inactive leucine, when compared with the leucine prepared from isovaleraldehyde, hydrogen cyanide and ammonia, was found to be identical with it, and further, both compounds gave d-leucine, when acted upon by the mould *Penicillium glaucum*, and the same leucic acid, when treated with nitrous acid. Leucine is therefore *α*-aminoisobutylacetic acid,



Among the syntheses of *α*-amino acids carried out by his own method by E. Erlenmeyer, jun., that of leucine was described in 1901 by Erlenmeyer and Kunlin. It was prepared from *α*-benzoylamido-*β*-isopropylacrylic acid,

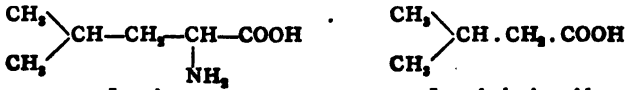


which resulted when isobutylaldehyde and hippuric acid were condensed together in the presence of acetic anhydride and the condensation product,



was treated with alkali.

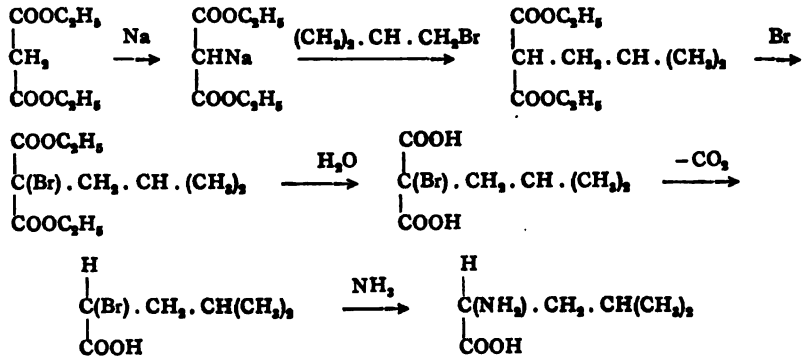
By heating *α*-benzoylamido-*β*-isopropylacrylic acid in sealed tubes at 150-170° C., with excess of ammonia, hydrolysis occurred with the formation of leucine, isovalerianic acid and benzoic acid :—



Leucine Isovalerianic acid

Bouveault and Locquin have also synthesised leucine by the reduction of *α*-oximinoisobutylacetic acid, which was prepared in a similar way to the isomeric compound from which they obtained isoleucine.

The most convenient method of preparing leucine by synthesis is that given by Fischer and Schmitz in 1906 by the action of ammonia upon the corresponding halogen derivative of isocaproic acid, which they prepared by brominating the alkylmalonic ester and heating, whereby it was converted into the bromo-fatty acid. The several reactions are represented by the following scheme :—

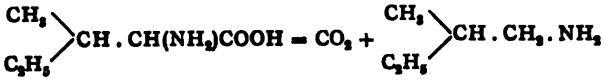

COOC2H5
CH2
COOC2H5
Na
COOC2H5
CHNa
COOC2H5
(CH3)2 . CH . CH2Br
COOC2H5
CH . CH2 . CH . (CH3)2
COOC2H5
Br
COOC2H5
C(Br) . CH2 . CH . (CH3)2
COOC2H5
H2O
COOH
C(Br) . CH2 . CH . (CH3)2
COOH
-CO2
H
C(Br) . CH2 . CH(CH3)2
COOH
NH3
H
C(NH2) . CH2 . CH(CH3)2
COOH

Isoleucine.

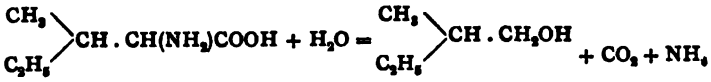
This amino acid was first obtained by F. Ehrlich in 1903 from the nitrogenous constituents of beet-sugar molasses, and was subsequently isolated by him from the decomposition products of fibrin and other proteins. Like leucine, to which it is very similar in properties, it thus appears to be a widely distributed constituent of the protein molecule.

Of the various isomeric amino-caproic acids only leucine and isoleucine occur in the protein molecule; both of them, combined with tyrosine and valine in the form of polypeptides from which they are easily split off by enzymes, seem to form a very important part of most proteins.

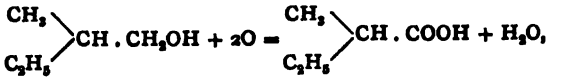
Ehrlich showed that leucine, when heated to 200° C., was converted into d-amylamine with loss of carbon dioxide:—



and that, when fermented by yeast in the presence of cane sugar, it yielded d-amylalcohol:—

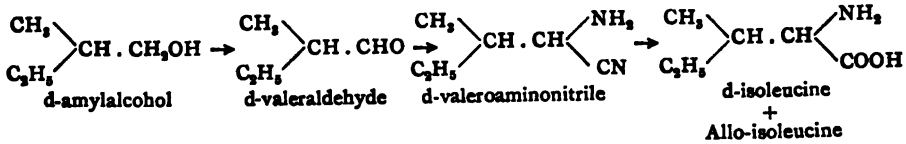


This was determined by oxidising to methylethylacetic acid:—

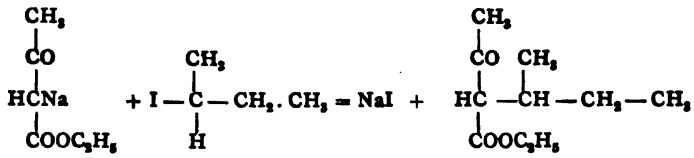


from which the constitution of isoleucine appeared to be α-amino-β-methyl-β-ethyl-propionic acid.

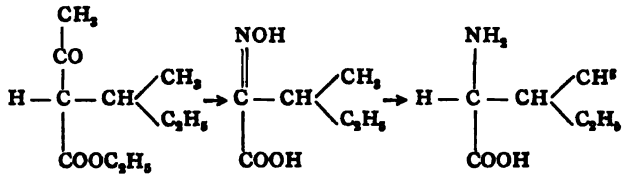
Ehrlich proved this by synthesising it from d-amylalcohol; this was first oxidised to valeraldehyde, which, on treatment with hydrogen cyanide and ammonia, gave valeroaminonitrile, and then, on hydrolysis, d-isoleucine mixed with allo-isoleucine; isoleucine has two asymmetric carbon atoms, and allo-isoleucine is formed by the rearrangement of the groups attached to one of them. By heating natural isoleucine with baryta water under pressure, it was found to also undergo a rearrangement, and the product seemed identical with that obtained by synthesis:—



Further proof that isoleucine has this constitution was given by Bouveault and Locquin in 1906. They synthesised it from sec. butyl-acetoacetic ester, which they prepared from sec. butyliodide and sodium-acetoacetic ester:—



This compound, when treated with nitrosyl sulphate was decomposed into acetic acid and *a*-oximino-sec-butylacetic acid, which, on reduction with zinc dust and hydrochloric acid in alcohol, gave a 60 per cent. yield of dl-isoleucine:—



Locquin has since obtained d-isoleucine from this racemic compound which was identical with Ehrlich's natural product, and this therefore has the above constitution.

By the same series of reactions which Fischer and Schmitz employed in the preparation of leucine, F. Ehrlich synthesised isoleucine in 1908 from malonic ester and secondary butyliodide, *i.e.*, according to the following scheme:—

$C_2H_5.CHI.CH_3 + CHNa(COOC_2H_5)_2 \rightarrow C_2H_5.CH(CH_3).CH(COOC_2H_5)_2$
$\rightarrow C_2H_5.CH(CH_3).CH(COOH)_2 \rightarrow C_2H_5.CH(CH_3).CBr(COOH)_2$
$\rightarrow C_2H_5.CH(CH_3).CHBr.COOH \rightarrow C_2H_5.CH(CH_3).CH(NH_2).COOH$

The same synthesis has also been carried out by Brasch and Friedmann.

Phenylalanine.

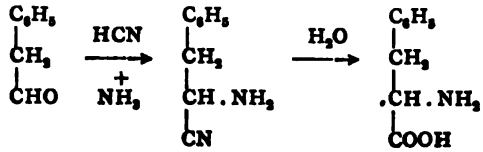
In a note published in 1879 Schulze mentioned a substance which he had obtained from the seedlings of *Lupinus luteus*; two years later he and Barbieri showed that this substance had the composition $C_9H_{11}NO_2$, and they described it as phenylamidopropionic acid, because, on oxidation, it gave benzoic acid, and, when heated, it lost carbon dioxide and gave a base, $C_8H_{11}N$. In its properties it closely resembled Tiemann's phenyl-aminoacetic acid, and they regarded it therefore as a homologue of this acid, though it differed from the substance described by Posen as phenyl-*a*-aminopropionic acid.

About the same time Schützenberger obtained a substance, which he called tyroleucine, by the action of baryta on proteins; when heated it gave a sublimate of aminovalerianic acid and a base $C_8H_{11}N$, which probably had as mother-substance the same body which was isolated by Schulze and Barbieri.

Schulze, Barbieri and Bosshard next showed that their substance arose during the germination of the seed, and that it was also obtained from vegetable proteins by hydrolysis, by hydrochloric acid and zinc chloride, or by baryta. It was therefore contained in the protein molecule.

It had been known for a long time that benzaldehyde and benzoic acid were formed by the oxidation of animal proteins, and that phenylpropionic and phenylacetic acids were products of putrefaction (Salkowski); phenylalanine was therefore regarded, as suggested by Tiemann, as the constituent from which these substances arose, but the actual presence of phenylalanine in the proteins was only proved when E. Fischer commenced his investigations upon them. He found that in some proteins it exceeded in amount that of tyrosine, and that it was in fact the principal aromatic constituent. The protein gelatin, which contains no tyrosine, and in which the presence of phenylalanine was demonstrated by Spiro, was found to contain this amino acid as its aromatic constituent.

The constitution of phenylalanine was determined in 1882 by Erlenmeyer and Lipp, who synthesised it by Strecker's method from phenylacetaldehyde, hydrogen cyanide and ammonia:—



This synthetical substance closely resembled Schulze and Barbieri's natural compound, and their identity was established. Posen's preparation, described under this name, was then regarded as phenyl-β-amino-propionic acid. It has since been shown to be phenyl-β-oxypropion-amide.

In 1893, a new method of synthesising amino acids, starting from hippuric acid, was introduced by Erlenmeyer jun., phenylalanine being the first product to be prepared.

When benzaldehyde is condensed with hippuric acid in the presence of acetic anhydride a Perkin's reaction takes place and benzoyl-α-amido-cinnamic acid is formed:—

$$C_6H_5.CHO + CH_2\begin{cases}NH.CO.C_6H_5\\COOH\end{cases} = C_6H_5.CH=C\begin{cases}NH.CO.C_6H_5\\COOH\end{cases} + H_2O$$

Under the influence of the acetic anhydride, this is converted into the azlactone,

$$C_6H_5.CH=C\begin{cases}N=C.C_6H_5\\CO—O\end{cases}$$

which, by hydrolysis by acids or by alkalies, is reconverted into benzoyl-α-amidocinnamic acid.

Benzoyl-α-amidocinnamic acid is reduced by sodium amalgam, or by zinc and hydrochloric acid, to benzoyl-α-amino-β-phenyl-propionic acid:—

$$C_6H_5.CH=C\begin{cases}NH.CO.C_6H_5\\COOH\end{cases} + H_2 = C_6H_5.CH_2.CH\begin{cases}NH.COC_6H_5\\COOH\end{cases}$$

from which the benzoyl group is easily removed by hydrolysis with the formation of phenylalanine:—

$$C_6H_5.CH_2.CH\begin{cases}NH.COC_6H_5\\COOH\end{cases} + H_2O = C_6H_5.CH_2.CH(NH_2).COOH + C_6H_5.COOH$$

Benzoyl-α-amidocinnamic acid is converted by the action of acids, or alkalies, into phenylpyruvic acid and benzamide,

$$C_6H_5.CH=C\begin{cases}NHCOC_6H_5\\COOH\end{cases} + HOH = C_6H_5.CONH_2 + C_6H_5.CH=C\begin{cases}OH\\COOH\end{cases}$$

$$\downarrow$$

$$C_6H_5.CH_2.CO.COOH$$

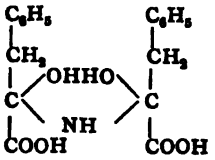
which Erlenmeyer proved by preparing the oxime and reducing it to phenylalanine[1]:—

$$C_6H_5.CH_2.CO.COOH \rightarrow C_6H_5.CH_2.C(NOH).COOH \rightarrow C_6H_5.CH_2.CH.NH_2.COOH$$

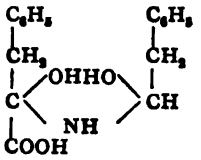
[1] Knoop and Hoessli find that the oxime of phenylpyruvic acid is most easily reduced to phenylalanine by aluminium amalgam.

Benzoyl-*a*-amidocinnamic acid is also converted by the action of ammonia into a compound, which yields phenylalanine on hydrolysis.

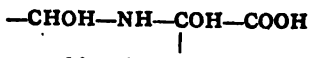
The mechanism of this reaction was explained by Erlenmeyer and Kunlin in 1899. Just as benzoyl-*a*-amidocinnamic acid is converted by alkali into phenylpyruvic acid and benzamide, so also does this reaction take place with ammonia; the phenylpyruvic acid then reacts with ammonia, giving a body of the composition,



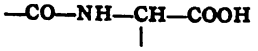
which loses carbonic acid, yielding,



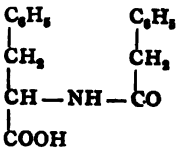
This substance contains the group,



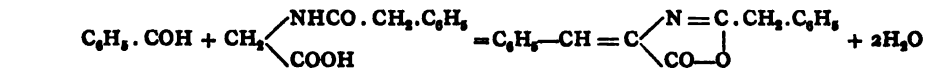
which, by rearrangement and by the loss of a molecule of water, becomes



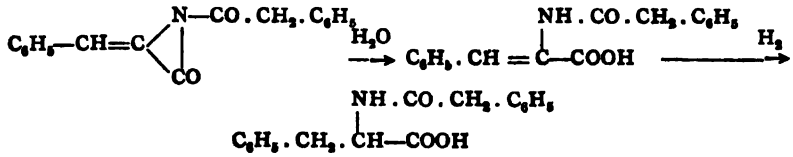
and it therefore changes into phenylacetyl-phenylalanine:—



Phenylalanine and phenylacetic acid result by subsequent hydrolysis. The proof of this reaction was given by the synthesis of phenylacetyl-phenylalanine by condensing benzaldehyde with phenaceturic acid:—



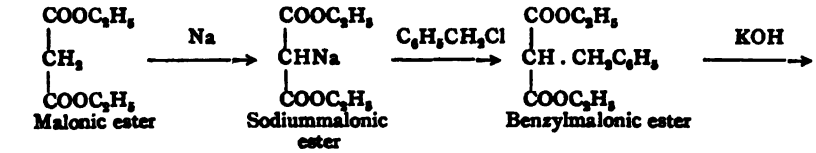
and hydrolysing the resulting azlactone, just as in the case of benzoyl-*a*-amidocinnamic acid, and reducing it with sodium amalgam:—

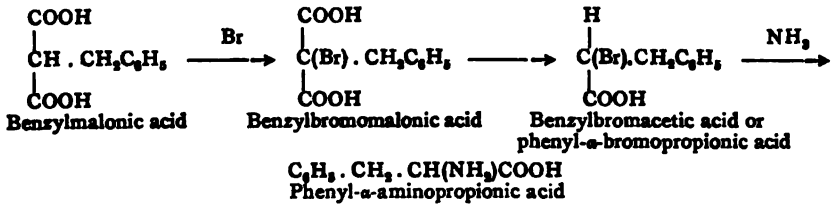


thus showing the identity of the two substances.

The condensation of benzaldehyde and hippuric acid, and the formation of phenylalanine by the action of ammonia had been previously carried out by Plöchl in 1884, but he was unable to explain the various stages which took place.

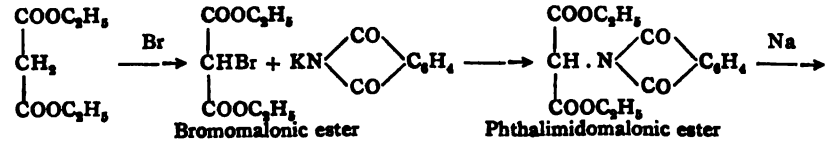
E. Fischer has synthesised phenylalanine by the action of ammonia upon the corresponding halogen fatty acid, which he prepares from malonic ester and benzylchloride. There are six stages in the complete process, which are as follow :—

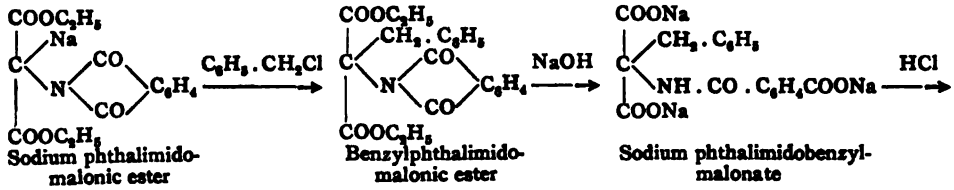


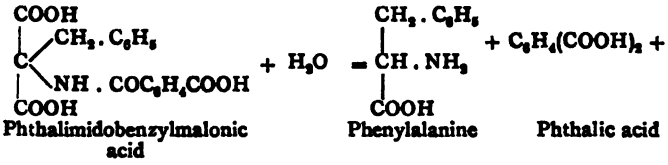


By this means large amounts of phenylalanine can be prepared ; they have been employed in studying the derivatives of phenylalanine and in the synthesis of polypeptides.

Another synthesis of phenylalanine from malonic ester, in which Gabriel's phthalimide reaction is also made use of, is described by Sörensen, namely,

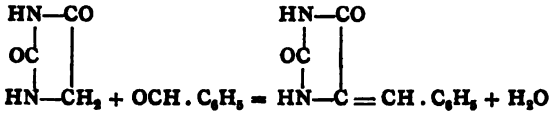




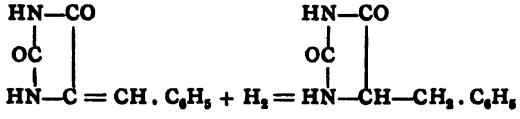


Phthalimidobenzylmalonic acid — Phenylalanine — Phthalic acid

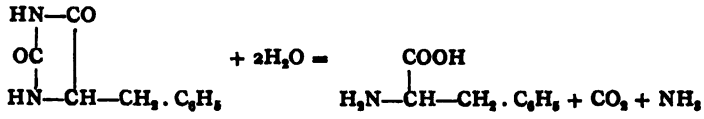
Wheeler and Hofmann, in 1911, showed that phenylalanine is very readily prepared from benzaldehyde and hydantoin. By the condensation of these compounds in acetic acid solution in presence of sodium acetate, benzalhydantoin is formed :—



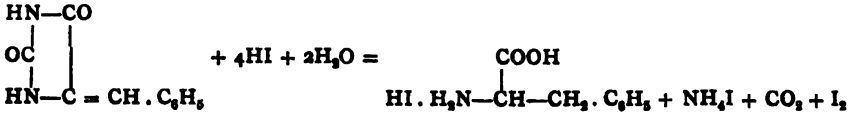
By the action of hydriodic acid this compound is reduced to benzylhydantoin,



which, on saponification with baryta, is converted into phenylalanine, carbon dioxide and ammonia :—



Benzalhydantoin is converted directly into phenylalanine hydriodide by boiling for several hours with hydriodic acid :—



Tyrosine.

By fusing cheese with caustic potash Liebig, in 1846, obtained a new compound, consisting of a mass of fine silky needles, soluble with difficulty in water; he named it tyrosine from τυρός, cheese. The same substance was isolated by Warren de la Rue from cochenille in 1848, and a year later Hinterberger obtained it by the hydrolysis of horn. Its presence in albumin, fibrin and caseinogen was demonstrated by Bopp.

The results of numerous investigations were published in 1860 by Städeler, who found tyrosine in silk-fibroin, mucin and various other proteins, and who also noted its occurrence in the free state in various animal organs, generally in conjunction with leucine. In plant seedlings it is also found in the free state. Since 1860, tyrosine has been constantly obtained from proteins by hydrolysis with acids and by the action of trypsin, and has long been regarded as a constituent of the protein molecule.

Its formula $C_9H_{11}NO_3$ was determined by Warren de la Rue and by Hinterberger. Strecker, in 1850, showed that it behaved like leucine and glycine, but pointed out that it did not belong to this series; and Wicke, in 1857, suggested that it stood in the same relation to the series of aromatic acids as glycine and leucine did to the fatty acids. Städeler was really the first to show that tyrosine was an aromatic compound, when he obtained chloranil (tetrachloroquinone) from it by the action of chlorine; he also found that it had a constitution similar to that of glycine and leucine. Fröhde also held this view, but Thudichum and Wanklyn, as they could not obtain picric acid from tyrosine by the action of nitric acid, considered that it was not an aromatic compound.

Städeler's discovery of the formation of chloranil from tyrosine led to the supposition that tyrosine was a derivative of salicylic acid, and on this assumption Schmidt and Nasse attempted to synthesise tyrosine from ethylamine and iodosalicylic acid, and from amidosalicylic and ethyl iodide, but did not succeed. On heating tyrosine they obtained a base $C_8H_{11}NO_2$, which they thought analogous to the one Schmidt had obtained by heating amidosalicylic acid; on this account they adhered to the theory that tyrosine was ethylamidosalicylic acid.

A great advance was made by Barth in 1865, who showed that tyrosine was not ethylamidosalicylic acid. As yet salicylic acid had

never been obtained from tyrosine, and Barth, in his attempt to prepare this compound from tyrosine by oxidation, by fusion with potash, obtained para-oxybenzoic acid and acetic acid, the decomposition taking place as follows:—

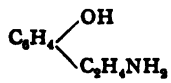
$$C_9H_{11}NO_3 + H_2O + O = C_7H_6O_3 + C_2H_4O_2 + NH_3.$$

He concluded that tyrosine was related to paracumaric acid,

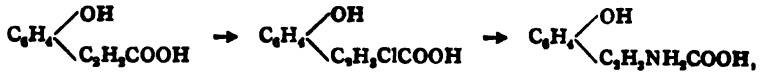
$$(OH)C_6H_4 . CH = CH . COOH,$$

in the same way that alanine was related to acrylic acid. Ost confirmed this result of Barth's several years later, when he obtained p-oxybenzoic acid by fusing tyrosine with caustic soda.

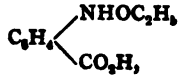
Tyrosine was now regarded as ethylamidopara-oxybenzoic acid; on reduction, therefore, it should yield ethylamine, but instead of this Hüfner, in 1868, obtained ammonia, and he supposed tyrosine to be amidophloretic acid.[1] This view was strengthened when Barth, in the following year, obtained p-oxybenzoic acid from phloretic acid and also from Schmidt and Nasse's base. This he regarded as



and tyrosine as oxyphenylamidopropionic acid, the nitrogen being attached to the side chain and not to the benzene ring as was supposed by Schmidt and Nasse. Barth's attempt to synthesise tyrosine from paracumaric acid by the following reactions,



which were also put forward by Beilstein and Kuhlberg, was not sufficiently successful to prove that tyrosine had this formula, so that Ladenburg, who stated that the reactions of tyrosine could be just as well explained by the formula,



synthesised this compound. It was quite different to tyrosine, and Barth's formula was therefore correct.

[1] Until 1900 phloretic acid was supposed to have the constitution,

$$OH . C_6H_4 . CH(CH_3) . COOH,$$

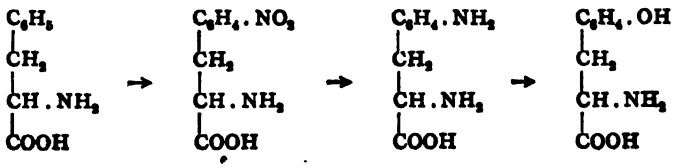
but in that year Bougault showed it to be β-oxyphenylpropionic acid,

$$OH . C_6H_4 . CH_2 . CH_2 . COOH.$$

The work of Baumann in 1879 upon the decomposition of tyrosine by putrefaction showed that hydroparacumaric acid, or p-oxyphenylpropionic acid, was the first product and that p-oxyphenylacetic and p-oxybenzoic acids were derived from this body. Bacterial decomposition can be carried as far as benzoic acid and benzene (Traetta-Mosca).

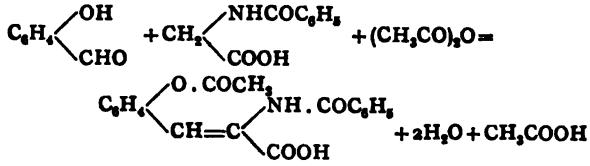
Tyrosine therefore must be p-oxyphenylaminopropionic acid. It only remained to determine the position of the NH_2 group, whether it was in the α- or β-position.

This was decided in 1882 by Erlenmeyer and Lipp who synthesised tyrosine from phenylalanine. Their first method, of preparing p-sulphonyl phenyl-α-aminopropionic acid and to exchange the sulphonic acid group for the hydroxyl group, was not successful, as in the fusion with potash the side chain also became oxidised and no tyrosine resulted. They then prepared p-nitrophenylalanine, and converted it into p-amidophenylalanine; on treating this latter compound with the calculated quantity of sodium nitrite and warming, they obtained p-oxyphenylalanine, thus,

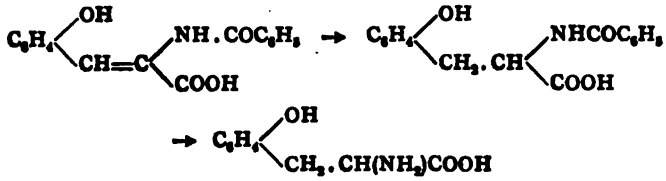


This compound had the same properties as the natural tyrosine, which was thus proved to be p-oxyphenyl-α-aminopropionic acid.

Erlenmeyer jun., and Halsey, in 1899, synthesised tyrosine by the condensation of hippuric acid with p-oxybenzaldehyde in the presence of acetic anhydride. The reactions are the same as those described by Erlenmeyer for the synthesis of phenylalanine, except that the hydroxyl group of the p-oxybenzaldehyde becomes acetylated in the process:—

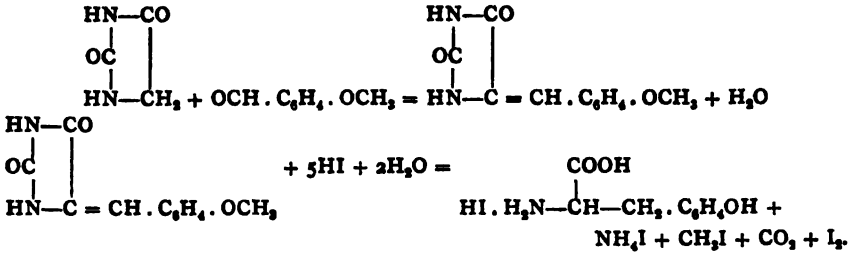


The azlactone is again formed, but, on hydrolysis by alkali, the acetyl group is removed and p-oxy-α-benzoylaminocinnamic acid is obtained. On reduction it yields benzoyltyrosine, from which tyrosine is formed by hydrolysis:—



Just as in the case of phenylalanine, p-oxy-*a*-benzoylaminocinnamic acid, when treated with ammonia, yields a substance which reacts with ammonia giving a complex compound; this, on hydrolysis, by heating in a sealed tube with hydrochloric acid, is converted into tyrosine.

Tyrosine is most conveniently prepared by synthesis from anisaldehyde and hydantoin. (Compare preparation of phenylalanine p. 97). Wheeler and Hoffmann have obtained an excellent yield in two operations :—

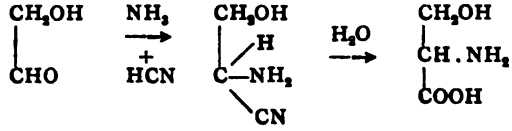


Tyrosine is obtained from the hydriodide by treatment with ammonia.

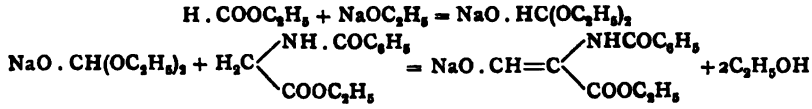
Serine.

Serine is, as yet, the only member of the oxyamino acids of the aliphatic series which has been isolated with certainty from the mixture of decomposition products of the proteins. It was first obtained in 1865 by Cramer from silk-gelatin, and was not again obtained until E. Fischer isolated it from the various proteins which he and his pupils have examined. Its occurrence in the free state, in human sweat, has been noticed by Embden and Tachau.

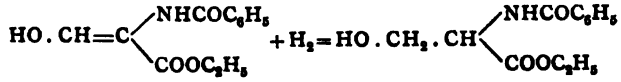
Cramer, its discoverer, showed that, when serine was treated with nitrous acid, it was converted into glyceric acid, and he recognised it as an aminolactic acid. It was regarded as *α*-amino-*β*-oxypropionic acid, but this was only definitely proved when it was synthesised by Fischer and Leuchs in 1902 from glycollic aldehyde, hydrogen cyanide and ammonia, which is the first instance of the employment of Strecker's method of building up oxyamino acids from oxyaldehydes:—



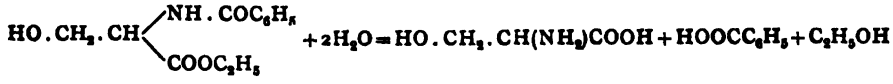
Serine is another of the amino acids which Erlenmeyer jun. synthesised in 1902 from hippuric acid, and which he described in detail with Stoop in 1904; by condensing formic ester and hippuric ester with sodium ethylate they obtained oxymethylene hippuric ester or formyl hippuric ester:—



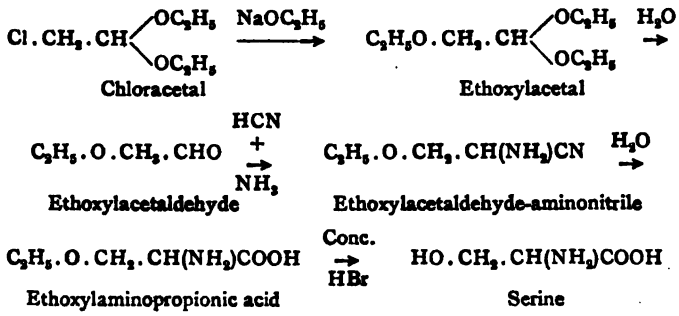
The free ester, obtained from the sodium salt as a thick oil, on reduction with aluminium amalgam gave N-benzoyl serine ester:—



which, when hydrolysed with dilute sulphuric acid, was converted into benzoic acid and serine:—



A better method of synthesising serine was described by Leuchs and Geiger in 1906, and was carried out as follows, starting from chloracetal:—



Cystine.

This compound was first described by Wollaston in 1810 under the name of cystic oxide, and was obtained from a urinary calculus. Lassaigne found it under the same conditions in a dog in 1823. Its presence in the kidney of an ox was shown by Cloetta in 1856, and in the following year Scherer found it in the liver of a patient, who had died from typhoid fever. The name cystine was given to it by Berzelius. Drechsel, in 1891, isolated it from horse's liver and in 1896 from a porpoise, and first regarded it as a normal product of metabolism. Arnold finds that cysteine, the reduction product of cystine, is present in all animal organs. In 1890 Külz obtained cystine by the digestion of fibrin with pancreas, and Emmerling, in 1894, found it mixed with tyrosine which he had prepared by the hydrolysis of horn. An attempt was made by Suter, in 1895, to obtain it from horn, but he could only obtain *a*-thiolactic acid, and it was not until 1899 that it was shown by K. A. H. Mörner to be a product of hydrolysis of this protein, and in 1901 also of other proteins. His results were confirmed by Embden, who was working independently and also obtained cysteine, which, as proved by Patten, is derived from cystine.

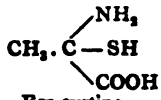
The earliest analyses of cystine are given by Prout, who overlooked the fact that cystine contained sulphur, the presence of which element was first shown by Baudrimont and Malagutis. Thaulow gave cystine the formula $C_6H_{12}N_2O_4S$, and pointed out that it was one of the few compounds made up of five elements. On account of the uneven number of atoms in its molecule, Gmelin replaced this formula by $C_3H_7NSO_2$, which formula was confirmed by Grote in 1864, and later by Külz in 1884.

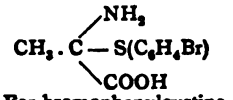
The first investigations on the constitution of cystine are those of Dewar and Gamgee in 1871, who, on treating cystine with nitrous acid, obtained an acid which they thought was pyruvic acid, $CH_3.CO.COOH$, and on this account gave cystine the constitution of

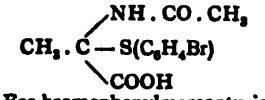
$$\begin{array}{l} CH_2(H_2N) \\ | \\ CS \\ | \\ COOH \end{array} \qquad \text{or } C_3H_7NSO_2$$

Hoppe-Seyler, as cited by Baumann and Preusse, showed that the nitrogen of cystine was separated off by alkalies as ammonia and not as methylamine, as would be expected from this formula, and he moreover maintained that the formula was $C_3H_7NSO_2$.

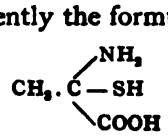
Baumann and Preusse's investigations in 1881 upon the fate of bromobenzene in the animal body, though they only constituted indirect evidence in regard to the constitution of cystine, were of great importance, as they were carried out at the time when cystine was a very scarce compound and only obtainable from calculi. They found that, when bromobenzene was given to animals, it was excreted in the urine in combination with a sulphur-containing compound, which combination had the formula $C_{11}H_{12}BrSNO_3$, and in this they were confirmed by Jaffé. When boiled with hydrochloric acid, this compound was converted into acetic acid and a substance $C_9H_{10}BrNSO_2$, from the empirical formula of which Baumann and Preusse supposed that it was cystine $C_3H_7NSO_2$, in which one of the hydrogen atoms was replaced by C_6H_4Br. Their further experiments led them to the conclusion that it was really a derivative of cystine. On decomposition by alkali, this latter compound yielded bromophenylmercaptan, ammonia and another substance, which they eventually recognised must be pyruvic acid. It had been shown that Dewar and Gamgee's formula for cystine, which was also based upon the formation of pyruvic acid, was not accurate, so they proposed the following:—


For cystine

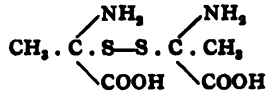

For bromophenylcystine


For bromophenylmercapturic acid

Baumann next found that cystine, on reduction with zinc and hydrochloric acid, was converted into a new base, which he called cysteine; this gave the same products on decomposition as cystine, into which it was easily reconverted by oxidation. He therefore recognised that these compounds were related to each other, as a mercaptan is to a disulphide; consequently the formula



was really that of cysteine, and that of cystine was

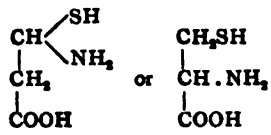


the former bromophenylcystine being bromophenylcysteine, etc.

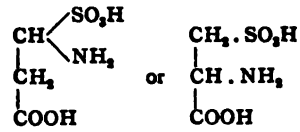
The actual formation of pyruvic acid from various mercapturic acids upon which these formulæ for cysteine and cystine were founded, was only shown later by Baumann's pupils, Königs, Brenzinger and Schmitz,

and in conjunction with Suter's observation that α-thiolactic acid was formed by the hydrolysis of horn, this formula for cystine was accepted. The results obtained, however, scarcely justified this formula as pointed out by Friedmann in 1902, who showed conclusively that the cystine, obtained from proteins, had not this constitution.

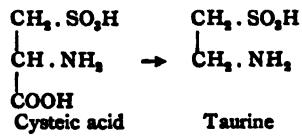
It had been found by Jochem in Hofmeister's laboratory that amino acids, when treated with nitrous acid in hydrochloric acid solution, were converted into the corresponding chloro-derivatives, and Friedmann, on applying this reaction to cystine obtained dichlorodithiopropionic acid; this when reduced gave β-thiopropionic acid, and on subsequent oxidation β-dithiopropionic acid, identical with the compound prepared from β-iodopropionic acid and potassium hydrogen sulphide. The sulphur atoms in cystine and cysteine were therefore in the β-position, and it remained to show in which position the amino group was situated, whether as



By oxidising cystine with bromine water, Friedmann obtained cysteic acid, *i.e.*, either



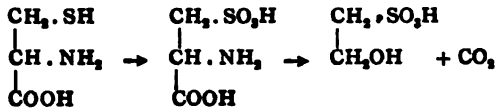
which, when heated, was converted by loss of carbon dioxide into taurine, which is only explainable by the second formula



Cysteic acid Taurine

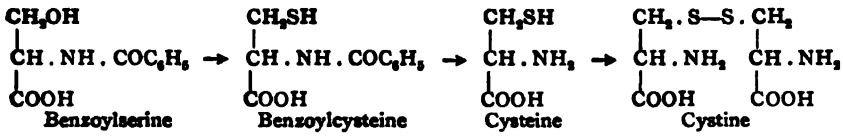
These reactions also showed how taurine might originate in the body from cystine.

At about the same time Neuberg, by treating cystine with nitric acid, obtained isethionic acid, which pointed to the correctness of Friedmann's formula; it at any rate showed that the sulphur and nitrogen atoms were attached to different carbon atoms. In the reaction the SH group was oxidised to the SO_3H group, and the NH_2 group was converted into the OH group by nitrous acid formed in the oxidation:—



The synthesis of cystine by Erlenmeyer jun. in 1903, which was more fully described by him and Stoop in 1904, showed that Friedmann's formula was correct:—

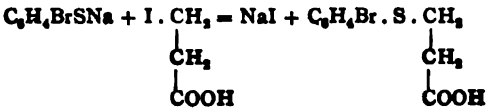
Benzoylserine was heated with phosphorus pentasulphide, and the product after hydrolysis with hydrochloric acid, gave cysteine which was converted by oxidation into cystine:—



Another synthesis of cystine was described by Fischer and Raske in 1908 (see page 148) which is similar to that of Erlenmeyer.

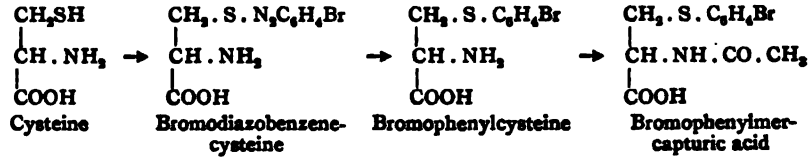
The formation of bromophenylmercapturic acid from bromobenzene and cystine in the organism, if it had the formula given it by Baumann now seemed scarcely possible, unless an isomeric *α*-thio-*β*-aminopropionic acid were also present in the protein molecule together with the di-*β*-thio-*α*-aminopropionic acid, or cystine. The investigation of their constitution was therefore taken up by Friedmann in 1904, who succeeded in showing that they were also derived from *β*-thio-*α*-aminopropionic acid and not from the isomeric *α*-thio-*β*-aminopropionic acid.

By the action of nitrous acid in hydrochloric acid solution on bromophenylcystine, prepared by Baumann's method, chlorobromophenylthiopropionic acid was obtained, which, on reduction, gave bromophenylthiolactic acid. This was identical with the substance prepared from *β*-iodopropionic acid and sodium bromophenylmercaptan:—



and therefore the SH group was in the *β*-position. Further proof was given by Friedmann by the synthesis of bromophenylmercapturic acid from cysteine. p-Bromodiazobenzene chloride was combined with cysteine; this compound, when decomposed by dilute soda, gave bromo-

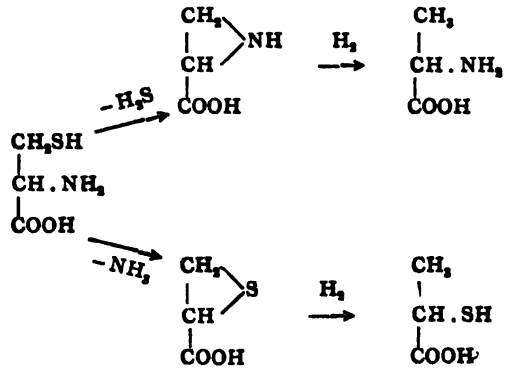
phenylcysteine, which, on acetylation, was converted into bromophenylmercapturic acid :—



It was first observed by Suter, in 1895, that *α*-thiolactic acid was formed by the hydrolysis of proteins, and it was constantly obtained by Friedmann. It was always regarded as a secondary product, but its formation from cystine could not be explained, as cystine is a *β*-thiopropionic acid.

In 1904 Mörner found that pyruvic acid was a constant product of hydrolysis of proteins, and that this compound gave *α*-thiolactic acid with hydrogen sulphide. Its formation was thus explained, but it was curious that hair, which is very rich in sulphur, gave less pyruvic acid than horn, which is less rich, and that caseinogen, which contains very little sulphur, also gave it. Mörner therefore supposed that there was another sulphur-containing compound in the protein molecule, which supposition was strengthened by Neuberg and Mayer's statement that stone cystine differed from protein cystine in many of its physical properties. Mörner's subsequent work on the decomposition of stone cystine, when he obtained *α*-thiolactic acid, ammonia and alanine, helped to support this idea; he regarded the alanine as formed from cystine and the *α*-thiolactic acid from the isomeric *α*-thio-*β*-aminopropionic acid, both of which he supposed were present in the stone cystine in equal quantities.

Fischer and Suzuki soon afterwards showed that Neuberg and Mayer's stone cystine contained tyrosine, and that its different behaviour to protein cystine was due to the presence of this compound. Rothera and also Abderhalden could find no difference between stone cystine and protein cystine, and further, Gabriel's synthesis of isocysteine or *α*-thio-*β*-aminopropionic acid and isocystine, which had quite different properties to cystine, though the two were much alike in many of their reactions, proved that stone cystine and protein cystine must be identical substances. Finally, it has been shown by Friedmann that *α*-thiolactic acid, ammonia and alanine can be obtained from protein cystine, which decomposition may take place, according to Gabriel, in the following way :—



Thus, the work of Friedmann on the constitution of cystine, its synthesis by Erlenmeyer jun. and by Fischer, definitely show that it has the composition

$$HOOC . CH(NH_2) . CH_2—S—S—CH_2 . CH(NH_2) . COOH.$$

The proof that bromophenylmercapturic acid is derived from cysteine, the formation of *a*-thiolactic acid from cystine derived either from stones or proteins, and the identity of protein cystine with stone cystine show that cystine is the sulphur-containing compound in the protein molecule.

The Sulphur in Proteins.

Although cystine is the only compound containing sulphur now known to be present in the protein molecule, yet numerous data have been collected which point to the presence of the element sulphur in some other compound than cystine. These data have been neglected since cystine was isolated and its constitution established, for its properties answered most of the questions which arose from the earlier work. Johnson, in 1911, drew attention to certain anomalies, and has made the suggestion that their explanation may be the presence of thioamide groups.

The presence of sulphur in proteins was a subject of very early investigation. It was commenced by Mulder, who was the first to observe that albumin, caseinogen, etc., when heated with alkali gave off hydrogen sulphide; in consequence of this he regarded these compounds as composed of sulphur and protein in various proportions. Fleitmann, a pupil of Liebig's, in 1847 then showed that this view of the constitution of albumin, etc., was erroneous, for he found that only a portion of the sulphur was split off by alkali, and that a portion still remained combined with the protein. The later investigators upon this question—Nasse, Danilewsky, Krüger, Suter, Malerba, Schulz—confirmed Fleitmann's results, and in addition they determined the ratio of total sulphur to loosely bound sulphur, as this, which was easily split off by alkali, was called. Their results varied considerably, which was due to the different methods they employed. In some proteins, *e.g.*, serumalbumin, the ratio of loosely bound sulphur to total sulphur was as 2 : 3, in others 1 : 2 or 3 : 5. Osborne found similar ratios for the large number of vegetable proteins which he examined. From these values determinations were made of the molecular weight: thus serumalbumin was given a molecular weight of 5,100, egg-albumin of 4,900, globulin of 4,600, edestin of 7,300.

If cystine be the only compound containing sulphur, the number of sulphur atoms in the protein molecule must be two or a multiple of two, then those proteins containing about 0·4 per cent. of sulphur must have a molecular weight of 15,000. The ratio of the sulphur to the iron in hæmoglobin and the data obtained by the depression of the freezing-point also pointed to a molecular weight of $(15{,}000)_n$. These conclusions could not be accepted, and Mörner's explanation of other sulphur-containing complexes in certain proteins, which yield hydrogen sulphide on boiling with alkali, was overlooked.

Mörner found that cystine, when boiled with alkali, yielded 75 per cent. of its sulphur as loosely bound sulphur and that, calculating the sulphur content of the keratins from the cystine he had isolated, the amount yielded by these proteins under the same conditions corresponded very closely; there were large discrepancies with other proteins. He also observed that sulphur was evolved from certain proteins, and other workers have frequently noticed the evolution of sulphur. If no other sulphur-containing compound be present in the proteins, Johnson's suggestion of the presence of thioamide groupings may be the explanation of the anomaly. Thioamides of amino acids are unknown, but Johnson is preparing compounds of this nature. We must hope that his results will fill up this gap in our knowledge.

B. Monoaminodicarboxylic Acids.

Aspartic Acid.

Asparagine, the amide of aspartic acid, was first isolated by Robiquet and Vauquelin, in 1806, from the juice of *Asparagus officinalis*; hence its name. Not only is asparagine found in asparagus, but also in the seedlings of lupines, peas, vetches, etc., from which it is best and most easily prepared. The natural compound is l-asparagine. The isolation of d-asparagine by Piutti has been proved by Pringsheim to be due to racemisation of the natural l-aspargine in the process of extraction, and the fact that the two isomers can be separated by fractional crystallisation.

Aspartic acid was first obtained by Plisson, in 1827, from asparagine by boiling it with lead hydroxide, and is usually prepared from this compound by hydrolysis with alkali, or acid.

Only however in 1868 was the presence of aspartic acid in vegetable proteins shown by Ritthausen, who obtained it by the hydrolysis of conglutin and legumin with sulphuric acid; in the following year Kreussler obtained it in the same way from animal proteins. In 1874 Radziejewski and Salkowski found that it was a product of the tryptic digestion of proteins, and the asparagine in plants most probably arises from the aspartic acid of the protein in the seed.

Its composition, $C_4H_7NO_4$, was established in 1833 by Boutron-Charlard and Pelouze, and confirmed by Liebig. In 1848 Piria showed that aspartic acid was converted into malic acid by the action of nitrous acid, and he regarded aspartic acid and asparagine as the two amides of malic acid

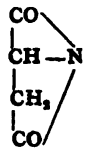
$$CONH_2.CH_2.CHOH.COOH \qquad CONH_2.CH_2.CHOH.CONH_2$$

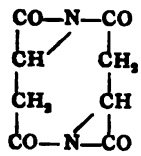
corresponding to oxamic acid and oxamide.

This idea of their constitution was proved to be erroneous by Kolbe in 1862, who showed that aspartic acid did not give off ammonia when boiled with dilute caustic alkali, and that asparagine only lost half of its nitrogen when thus treated. Aspartic acid was therefore not the amide of malic acid, but amino-succinic acid, and asparagine was the amide of this compound.

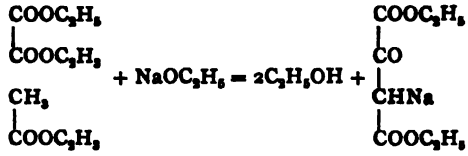
The first synthesis of aspartic acid is that by Dessaignes in 1850, who obtained a crystalline substance by heating acid ammonium malate to 160-200° C., which, when treated with hydrochloric or nitric acid, was converted into aspartic acid. In the same way Dessaignes ob-

tained aspartic acid from acid ammonium fumarate and maleate. At Liebig's suggestion these reactions were confirmed by Wolff. It was shown by Engel, in 1887, that aspartic acid could be obtained directly by heating maleic or fumaric acid with alcoholic ammonia to 140-150° without the formation of the intermediate substance which is fumarimide, and to which the following constitutional formulæ have been given :—

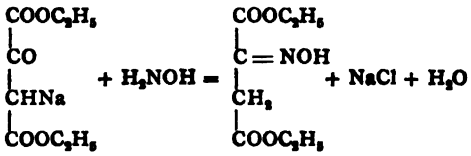




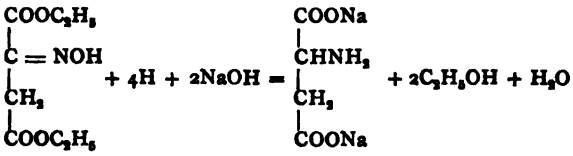
These syntheses give no indication as to the structure of aspartic acid, the constitutional formula of which is based upon Kolbe's work, that it is amino-succinic acid; the only synthesis of aspartic acid which confirms this constitution appears to be that by Piutti in 1887. Sodium oxalacetic ester, prepared from oxalic ester and acetic ester in the presence of sodium ethylate :—



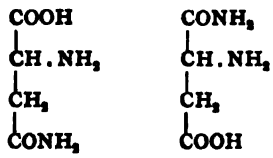
gives an oxime when treated with hydroxylamine hydrochloride :—



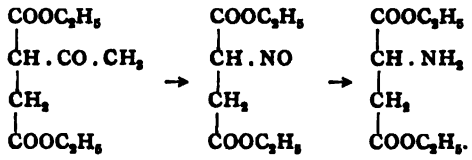
and this is reduced by sodium amalgam to sodium aspartate :—



From this oxime Piutti has also prepared the two isomeric asparagines :—



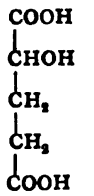
Schmidt and Widmann have prepared the ester of aspartic acid from acetyl-succinic ester. This was converted by the action of nitrous acid into the nitroso-derivative, which, on reduction with zinc and acetic acid, gave aspartic ester :—



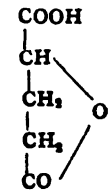
Glutamic Acid.

The presence of this amino acid in the protein molecule was shown by Ritthausen in 1866, who obtained it from wheat gluten by hydrolysis with sulphuric acid. He showed that it was an amino acid and termed it glutamic acid, on account of its first preparation from gluten. Subsequently Ritthausen and Kreussler isolated it from the hydrolysis products of other vegetable proteins. Kreussler could not demonstrate its presence in animal proteins, in which it was afterwards shown to occur in 1873 by Hlasiwetz and Habermann. Not only is glutamic acid formed by acid hydrolysis, but also by the action of enzymes: Knieriem and Kutscher obtained it by the tryptic digestion of fibrin, and its amide, glutamine, is found in the extracts of seedlings, as shown by Schulze, v. Gorup-Besanez and Scheibler.

Ritthausen gave glutamic acid the empirical formula $C_5H_9NO_4$, and found that, when treated with nitrous acid, it was converted into an oxyacid, which he termed glutanic acid and regarded as a homologue of malic acid. Dittmar again prepared glutanic acid and reduced it with hydriodic acid to an acid which was shown by Markownikoff to be what we now call glutaric acid; this was identical with the substance obtained by the hydrolysis of trimethylene cyanide, which was prepared from trimethylene bromide $CH_2Br . CH_2 . CH_2Br$ and potassium cyanide. Glutanic acid differed from Simpson's β-hydroxy-glutaric acid, and Markownikoff regarded it as the α-hydroxy-glutaric acid,

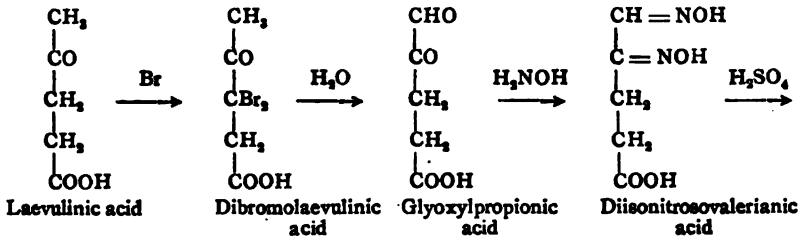


which, according to Bredt, exists in the free state as the γ-lactone.

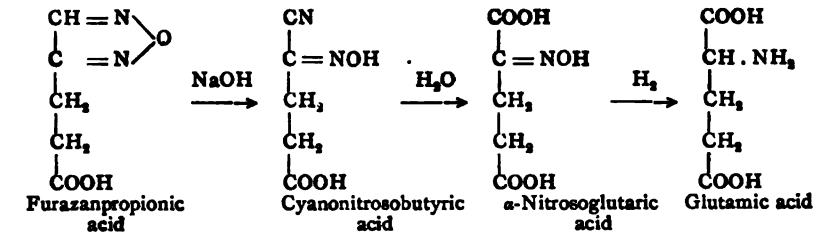


Glutamic acid would therefore be α-aminoglutaric acid. The proof

for this constitution was only given in 1890 by L. Wolff who synthesised glutamic acid from laevulinic acid. Dibromolaevulinic acid is obtained by bromination, and this when boiled with water gives diacetyl and glyoxylpropionic acid; diisonitrosovalerianic acid is formed from the latter, on treatment with hydroxylamine:—

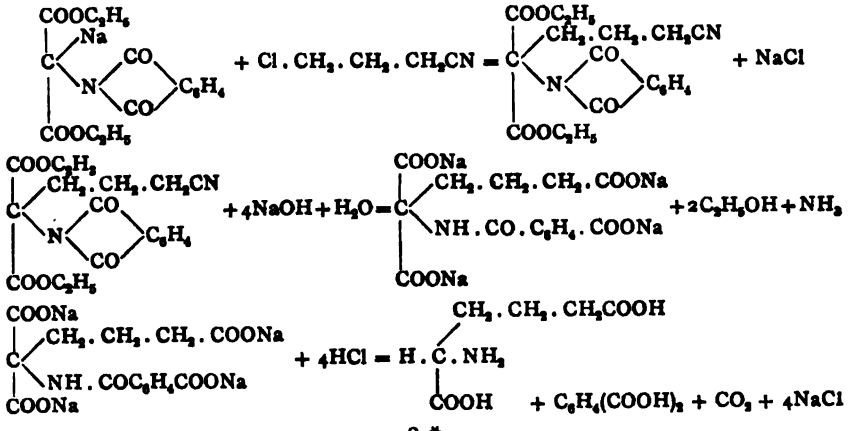


This is converted into furazanpropionic acid by sulphuric acid and then into cyanonitrosobutyric acid by caustic soda. Saponification changes the cyanonitrosobutyric acid into *a*-nitrosoglutaric acid from which glutamic acid is obtained by reduction:—



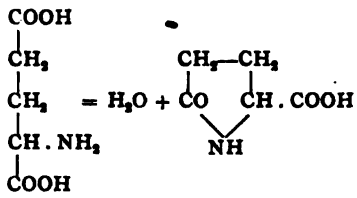
This appears to be the only recorded synthesis of glutamic acid.

The next member of this homologous series, *a*-amino-adipic acid, has been prepared by Sörensen from phthalimidosodium malonic ester and chlorobutyronitrile in the following way:—



Sörensen suggests that the same reactions might be employed for the synthesis of aspartic acid and of glutamic acid, in the case of the former by condensing the sodium phthalimidomalonic ester with chloracetic ester and in that of the latter with β-chloropropionic ester, $Cl.CH_2.CH_2.COOC_2H_5$. In view of our having only one synthesis of each of these two amino acids, and that these syntheses are somewhat arduous, Sörensen's suggestion might with advantage be carried out.

Glutamic acid, as also its salts and esters, is very easily converted, when heated, into its anhydride, pyrrolidone carboxylic acid :—



and *vice versa*, pyrrolidone carboxylic acid is easily reconverted into glutamic acid by hydrolysis with acids or alkalies.

On account of this ready interconversion of the one compound into the other there is the possibility that pyrrolidone carboxylic acid is present in the protein molecule in the place of glutamic acid, or at any rate in place of part of the glutamic acid.

Though this possibility does not seem likely in the case of those proteins examined by Osborne, Leavenworth and Brautlecht, who found that the amount of amide nitrogen corresponded very closely with the amount of dibasic monamino acid, yet in other proteins pyrrolidone carboxylic acid may be present. The method of hydrolysis, which would in any case yield glutamic acid, gives no clue, but a clue may be obtained if pyrrolidone carboxylic acid is formed by the action of enzymes. This question is under investigation by Abderhalden and Kautzsch, who are devising a method for their separation. Glutamic acid differs from pyrrolidone carboxylic acid in forming a carbamino acid. (See Part II.)

The easy formation of a pyrrole ring compound from glutamic acid, which occurs so widely in nature, suggests that the pyrrole ring in chlorophyll and hæmoglobin may arise from this compound. The reduction to proline has been effected by Fischer and Boehner.

C. Diaminomonocarboxylic Acids.

Drechsel's discovery of lysine amongst the products of hydrolysis of caseinogen in 1889 first showed that the monoamino acids were not the only constituents of the protein molecule; the substance, lysatinine, which he and his pupils also isolated a few years later from several proteins, was shown by Hedin to be a mixture of arginine and lysine, the former body having been many years previously obtained by E. Schulze and E. Steiger from germinating seedlings. Though ornithine had been discovered more than ten years before lysine, its importance as a constituent of the protein molecule was not recognised until it was shown by Schulze to be contained in arginine. Histidine, discovered in 1896 by Kossel, was classed, until its constitution was determined, with the diamino acids on account of its method of separation and its close relationship in many of its properties to arginine and lysine, the three bases having been termed by Kossel the hexone bases and regarded as a very important portion of the protein molecule.

The synthesis of the diamino acids, in comparison with that of the monoamino acids, is very much more difficult and has only been achieved within the last few years.

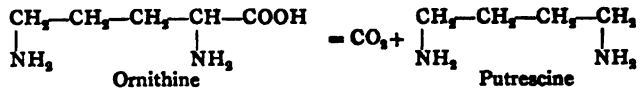
Diamino-acetic Acid.—This acid, the first member of the series, was described by Drechsel as a decomposition product of caseinogen. Its existence is extremely doubtful, its attempted synthesis by Klebs did not succeed, and Willstätter could only obtain certain of its derivatives.

Diaminopropionic Acid has not yet been described as a constituent of the proteins, but it was synthesised by Klebs in 1894 by the action of ammonia upon dibromopropionic acid.

Diaminobutyric Acid.—α-γ-Diaminobutyric acid was prepared in 1901 by E. Fischer by the same method as he employed in the synthesis of ornithine.

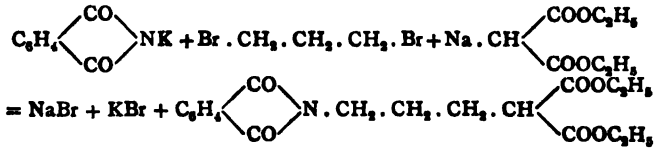
Ornithine or α, δ-diaminovalerianic Acid.—In 1877 Jaffé obtained from the urine of birds, which he had fed with benzoic acid, dibenzoyl ornithine or ornithuric acid, and from this substance he prepared ornithine chloride. He regarded it as a diaminovalerianic acid, the first known representative of the series of diamino acids, but only in 1898 was the position of the two amino groups definitely determined by Ellinger, who obtained putrescine from it by putrefaction; the identity of putrescine with tetramethylenediamine had been previously shown by Udransky and Baumann, and ornithine was therefore α, δ-diamino-

valerianic acid, the hydrolysis of ornithine taking place according to the equation:—

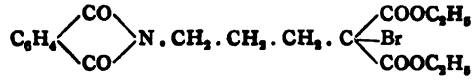


The expected synthesis of *a*, δ-diaminovalerianic acid, which was attempted by Willstätter in 1900, by the action of ammonia upon *a*, δ-dibromovalerianic acid, led to the synthesis of *a*-pyrrolidine-carboxylic acid, and it was in the following year that the synthesis of this important naturally occurring diamino acid was accomplished by E. Fischer. He made use of Gabriel's phthalimide method with a slight modification and obtained ornithine by the following series of reactions:—

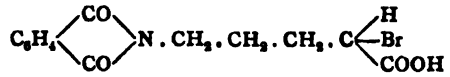
γ-phthalimidopropylmalonic ester was prepared from potassium phthalimide, propylene bromide and sodium malonic ester:—



On bromination this gave phthalimidopropylbromomalonic ester,



which, on treatment with ammonia, did not give the desired result. On hydrolysis, however, and by loss of carbon dioxide, it is converted into δ-phthalimido-*a*-bromovalerianic acid,



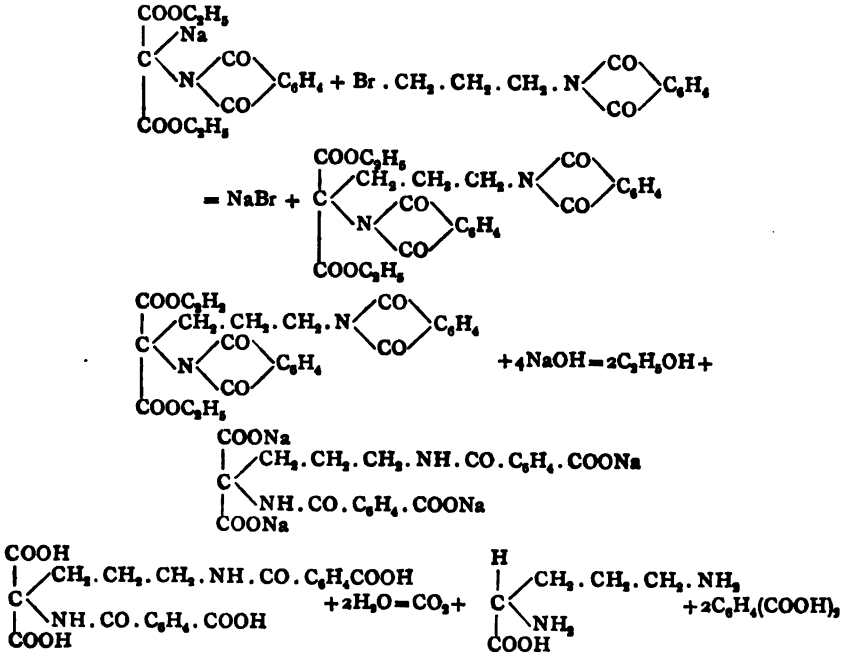
The Br atom is exchanged for the NH_2 group by treatment with ammonia, and on subsequent hydrolysis, this acid yielded *a*, δ-diamino-valerianic acid or ornithine,

$$H_2N . CH_2 . CH_2 . CH_2 . CH(NH_2) . COOH.$$

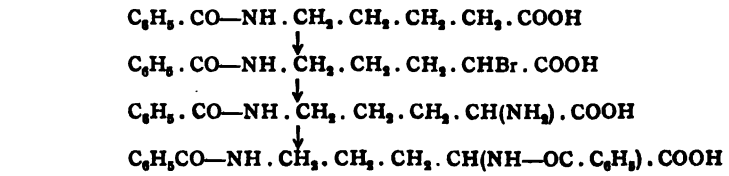
The dibenzoyl compound only differed from Jaffé's ornithuric acid by being optically inactive.

By a very similar series of reactions Sörensen has also synthesised ornithine: he first introduces the phthalimido group into the sodium malonic ester and then allows γ-bromopropylphthalimide to act upon this; the new substance is treated with sodium and alcohol, and, on

subsequent hydrolysis of the acid, whereby the phthalyl groups are removed and by loss of carbon dioxide, it yields ornithine, thus:—



An easier synthesis of ornithine was described by Fischer and Zemplen in 1909. Benzoyl-δ-aminovalerianic acid is prepared by the oxidation of benzoylpiperidine with permanganate. This compound is converted by the action of bromine and phosphorus into benzoyl-δ-amino-α-bromovalerianic acid, which, on treatment with ammonia, gives monobenzoylornithine. The inactive ornithuric acid (dibenzoyl-α, δ-diaminovalerianic acid) is then easily made by benzoylation :—



Arginine.

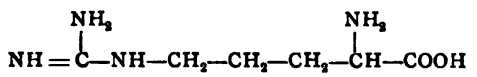
In 1886 E. Schulze and E. Steiger obtained a nitrogenous base from the extracts of the germinated cotyledons of *Lupinus*, which had the composition $C_6H_{14}N_4O_2$, and to which they gave the name arginine; it was also found in the seedlings of other plants and is contained in all the vegetable proteins. Shreiner and Shorey have found it in soil.

Hedin, in 1894, isolated it from the products of hydrolysis of horn, gelatin, conglutin, vitellin, egg-albumin, blood-serum, caseinogen. He also showed that Drechsel's lysatinine consisted of a mixture of arginine and lysine. From elastin both Bergh and Hedin failed to isolate it, but its presence in this protein was demonstrated by Kossel and Kutscher. Its occurrence in the protamines was shown by Kossel in 1896, and in histone from leucocytes by Lawrow in 1899. About the same time Kutscher found that it was contained in antipeptone, obtained by the tryptic digestion of fibrin; it is also formed when protamines are digested by trypsin (Kossel and Matthews). It is present in the free state in ox spleen, fish-flesh and bull's testis (Totani and Katsuyama).

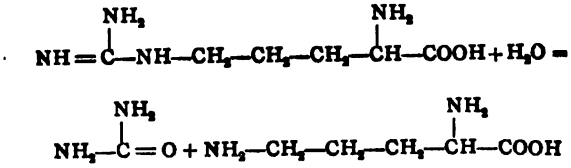
The identity of the arginine obtained from animal proteins with that from vegetable proteins was at first denied by Gulewitsch, but a little later he showed that they were identical, as also did Schulze.

The arginine, as it occurs in the proteins, is the dextro-rotatory modification except in fibrin, from which both Kutscher and Cathcart have isolated the inactive form.

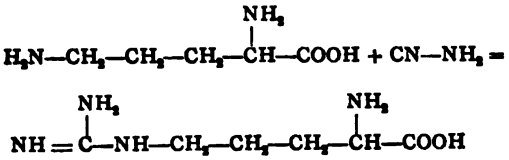
Schulze and Likiernik, in 1891, found that urea was formed when arginine was heated with baryta; they therefore supposed that arginine was a derivative of guanidine. In 1897 Schulze and Winterstein found that ornithine was also formed; they isolated it as its dibenzoyl derivative, which was found to be identical with Jaffé's ornithuric acid. Ornithine was regarded as a diaminovalerianic acid; Schulze and Winterstein showed that it contained two NH_2 groups not attached to neighbouring carbon atoms, and suggested that arginine, as it was a derivative of guanidine and ornithine, might have the following constitution:—



and that the formation of urea and ornithine might be explained according to the equation:—

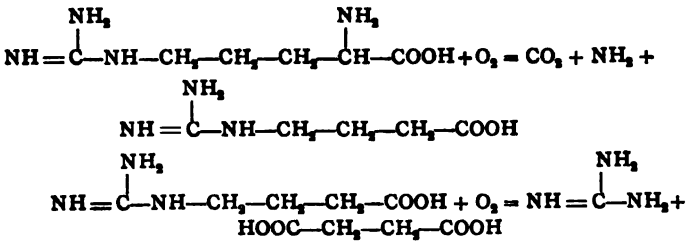


Schulze and Winterstein, in 1899, showed that arginine could be obtained by synthesis from cyanamide and ornithine:—



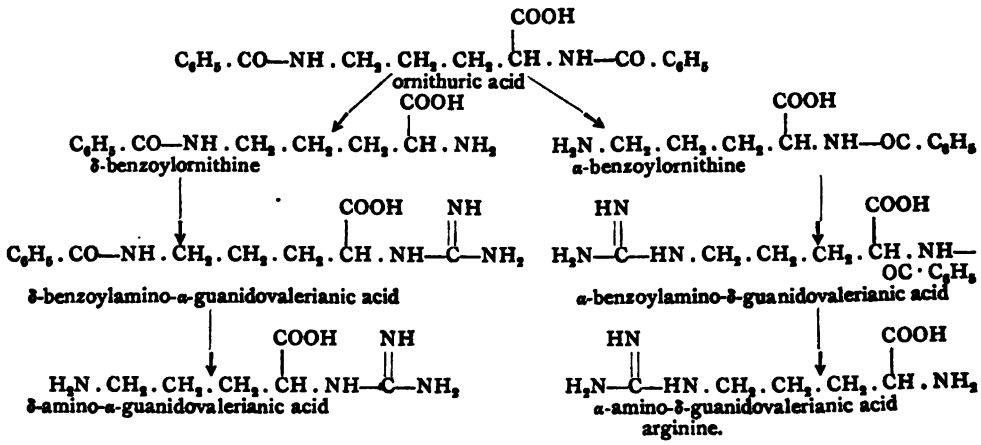
This synthesis proved that arginine contained a guanidine group.

The presence of a guanidine group in arginine is also proved by the formation of guanidinebutyric acid and of guanidine and succinic acid by oxidation with permanganate, which probably takes place according to the following equations (Kutscher):—



Neither the synthesis of arginine by Schulze and Winterstein nor the oxidation experiments of Kutscher prove to which of the amino groups of ornithine the guanido group is attached; the δ-position was assumed to be the more probable.

This constitution of arginine was only established by Sörensen, in 1910, who prepared the two isomers. He found that ornithuric acid (dibenzoylornithine) gave δ-monobenzoylornithine, when hydrolysed with concentrated hydrochloric acid, and *a*-monobenzoylornithine, when hydrolysed with baryta water. By the addition of cyanamide to these two compounds he obtained δ-monobenzoylamino-*a*-guanido-valerianic acid and *a*-monobenzoylamino-δ-guanidovalerianic acid. The benzoyl group was then removed by boiling with hydrochloric acid:—



The resulting *α*-amino-δ-guanidovalerianic acid was found to be identical with racemic arginine.

Lysine.

Lysine was discovered by E. Drechsel amongst the decomposition products of caseinogen in 1889, and its presence in other proteins—gelatin, egg-albumin, conglutin, fibrin—was shown by his pupils, Ernst Fischer, Siegfried and Hedin. It was found by Kutscher in antipeptone and by Kossel in the protamines. Its occurrence in germinating seedlings was demonstrated by Schulze and in vegetable proteins by Schulze and Winterstein. Thus, like arginine and histidine it is a very widely occurring constituent of the proteins.

Drechsel gave it the formula $C_6H_{14}N_2O_2$ and regarded it as a diaminocaproic acid; Ellinger proved in 1899 that it possessed this constitution, by obtaining cadaverine from it by putrefaction, which showed that the two amino groups were in the *a*, *e*-positions:—

$$NH_2.CH_2.CH_2.CH_2.CH_2.CH(NH_2)COOH = CO_2 + NH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.NH_2.$$

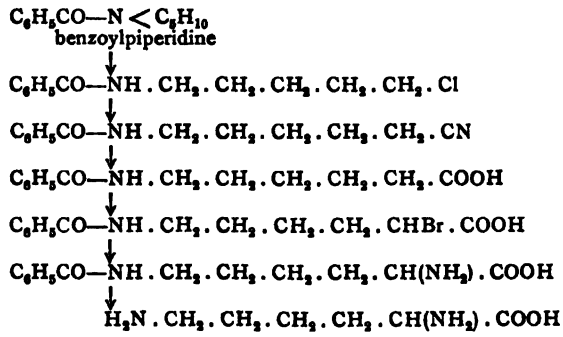
Henderson's experiments also showed that lysine must have this constitution, namely *a*, *e*-diaminocaproic acid. Its constitution was only definitely determined by synthesis by Fischer and Weigert by the following method:—

When γ-cyanopropylmalonic ester is treated with nitrous acid, it loses one of its carboxethyl groups and is converted into *a*-oximido-δ-cyanovalerianic acid, which on reduction with sodium amalgam yields *a*, *e*-diaminocaproic acid, thus:—

$$NC.CH_2.CH_2.CH_2.CH\langle^{COOC_2H_5}_{COOC_2H_5} \rightarrow NC.CH_2.CH_2.CH_2.C(=NOH).COOC_2H_5 \rightarrow H_2N.CH_2.CH_2.CH_2.CH_2.CH(NH_2).COOH$$

Sörensen has also prepared lysine by the same method which he employed for the synthesis of ornithine.

Neither of these syntheses are very suitable for the preparation of lysine. A good yield of lysine is more easily obtained by v. Braun's method. Benzoylpiperidine is converted by the action of phosphorus pentachloride into benzoylchloramylamine; the chlorine atom is exchanged for the CN group which is hydrolysed to the COOH group. The resulting *e*-benzoylaminocaproic acid is brominated with bromine and phosphorus. Treatment with ammonia gives *e*-benzoylamino-*a*-aminocaproic acid from which lysine is obtained by hydrolysis. The reactions are:—

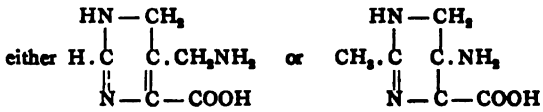

C6H5CO—N < C5H10
benzoylpiperidine
C6H5CO—NH . CH2 . CH2 . CH2 . CH2 . CH2 . Cl
C6H5CO—NH . CH2 . CH2 . CH2 . CH2 . CH2 . CN
C6H5CO—NH . CH2 . CH2 . CH2 . CH2 . CH2 . COOH
C6H5CO—NH . CH2 . CH2 . CH2 . CH2 . CHBr . COOH
C6H5CO—NH . CH2 . CH2 . CH2 . CH2 . CH(NH2) . COOH
H2N . CH2 . CH2 . CH2 . CH2 . CH(NH2) . COOH

D. Heterocyclic Compounds.

Histidine.

Histidine was discovered in 1896 by Kossel amongst the decomposition products of sturine, the protamine obtained from the ripe testis of the sturgeon. In the same year Hedin isolated a base from the products of hydrolysis of various proteins, which he regarded as identical with Kossel's histidine, and this was subsequently shown to be the case by Kossel and Kutscher. Kutscher also found it in antipeptone obtained by the pancreatic digestion of fibrin, and Schulze and Winterstein have shown that it occurs as a decomposition product of various vegetable proteins. Its presence in thyreoglobulin was shown by Koch.

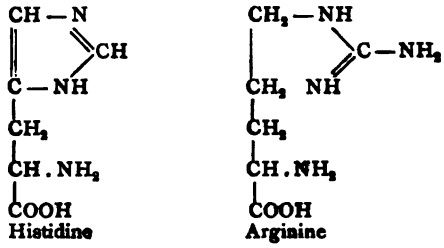
Histidine was found to possess the formula $C_6H_9N_3O_2$, but beyond the facts that it formed a dichloride, that two of its hydrogen atoms were replaceable by metals and that it was optically active and therefore contained an asymmetric carbon atom, no experiments to determine its constitution were published until 1903. Herzog then showed that it gave the biuret reaction on warming, that it did not contain a methyl nor a methoxyl group, and that it was very resistant to oxidising reagents and, in fact, behaved as a saturated compound. At the same time Fränkel showed that it contained a carboxyl group and an amino group, which was replaced by the hydroxyl group by the action of nitrous acid; it was therefore $(NH_2).C_5H_6N_2.COOH$. As it gave Weidel's pyrimidine reaction and did not contain a pyrrole ring nor a guanidine group, Fränkel suggested that it might be a derivative of dihydropyrimidine,



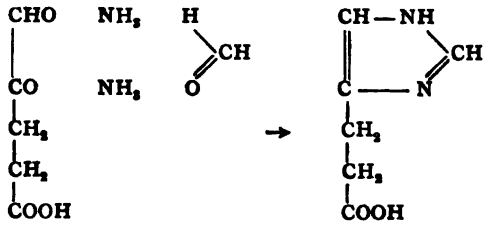
but Weigert pointed out that neither of these formulæ possessed an asymmetric carbon atom, and that histidine was optically active; consequently its formula must remain as $(NH_2).C_5H_6N_2.COOH$.

Pauly, in 1904, confirmed the presence of a carboxyl group, and showed that histidine contained a secondary amine group as well as a primary amine group by preparing a dinaphthalene sulphonyl derivative, the remaining nitrogen atom being probably a tertiary one. He pointed out that the resistance of histidine to oxidation and to acid permanganate, and that the formation of a di-silver compound were against the presence of a dihydropyrimidine ring in its molecule. These properties, as well as the capability which histidine possessed of forming azo-dyes with diazonium salts, pointed to the existence of a glyoxaline or

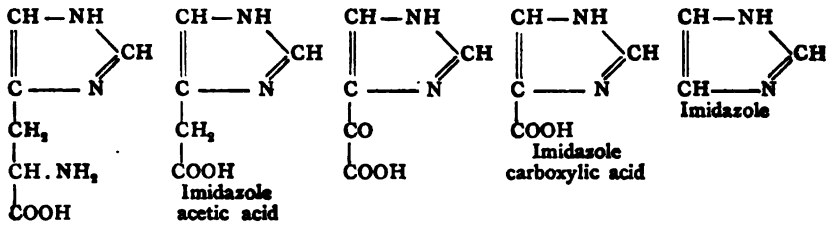
imidazole ring in its composition. Pauly, therefore, gave it the constitution of imidazole-amino-propionic acid, at the same time showing its relation to arginine :—



This assumption of Pauly's was confirmed by Knoop and Windaus, who found that histidine is resistant to reduction by sodium and alcohol whereas the pyrimidine ring is very unstable towards this reagent. On reducing Fränkel's oxydesaminohistidine, which is obtained from histidine by the action of nitrous acid, they obtained β-imidazole-propionic acid.[1] This compound was identical with the synthetical product prepared from glyoxylpropionic acid, ammonia and formaldehyde :—



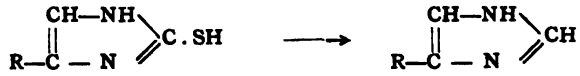
The presence of an imidazole ring in histidine was thus proved, and it only remained to show the position of the amino group. Fränkel urged certain objections against the presence of an imidazole ring in histidine, but Knoop and Windaus showed that these did not hold good. Knoop has since obtained imidazole-glyoxylic acid, imidazole-carboxylic acid, and imidazole from oxydesaminohistidine, and also imidazole-acetic acid. The imidazole ring is therefore in the β-position and histidine is β-imidazole-α-amino-propionic acid :—



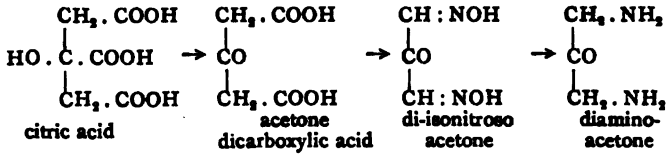
[1] Windaus and Vogt in 1908 showed that Fränkel's chlorohistidine carboxylic acid was the hydrochloride of β-imidazole-propionic acid.

A suggested synthesis of histidine in 1909 by Gerngross from *a*-methyl imidazole and chloral was unsuccessful. The reaction, according to Windaus, proceeds in a different manner.

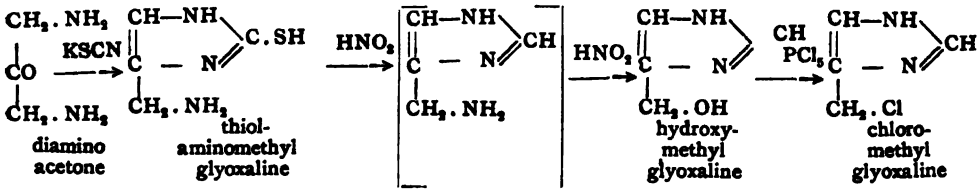
Histidine, and also its natural isomer, were synthesised by Pyman in 1911. By making use of Gabriel's method of synthesising the glyoxaline or imidazole ring, which consists in the condensation of aminoketones, $R.CO.CH_2NH_2$, with potassium thiocyanate and the subsequent oxidation of the thiol group with nitric acid:—



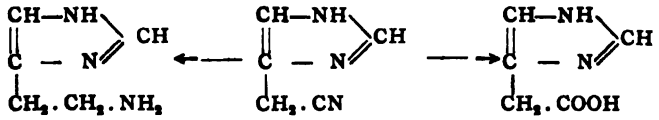
and of Kalischer's method of preparing diaminoacetone from citric acid:—



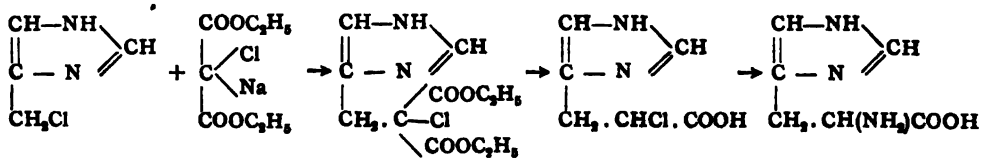
Pyman obtained thiol-aminomethyl glyoxaline. Nitric acid removed the thiol group as sulphuric acid and at the same time the nitrous acid formed in the reaction attacked the amino group replacing it by the hydroxyl group and giving hydroxymethyl glyoxaline. Chloromethyl glyoxaline was then prepared by the action of phosphorus pentachloride:—



The chlorine atom of chloromethyl glyoxaline was easily replaceable; by exchanging it for the CN group by treatment with potassium cyanide and hydrolysing the nitrile glyoxaline acetic acid (Knoop's imidazole-acetic acid) was obtained; by reducing the nitrile imidazolyl-ethylamine or glyoxaline ethylamine, a base found by Barger and Dale to be one of the active principles of ergot and derived from histidine, was obtained:



By treating the chloromethyl glyoxaline with ethyl sodiochloromalonate, ethyl glyoxaline chloromalonate was formed. This ester on saponification lost carbon dioxide and gave glyoxaline chloropropionic acid; by treating this compound with ammonia the chlorine atom was replaced by the NH_2 group and glyoxaline amino propionic acid, or histidine was obtained :—



chloromethyl glyoxaline. | ethyl sodiochloromalonate. | ethyl glyoxaline chloromalonate. | glyoxaline chloropropionic acid. | glyoxaline amino propionic acid or histidine.

The racemic histidine, thus obtained, was resolved into its optically active isomers by the fractional crystallisation of its salt with d-tartaric acid.

Glyoxaline-chloropropionic acid only differed from Windaus and Vogt's compound and glyoxaline-oxypropionic acid, prepared by the action of silver oxide, only differed from Fränkel's oxydesaminohistidine in their melting-points which is most probably due to the fact that the compounds of Windaus and Vogt and of Fränkel were prepared from the natural l-histidine.

In connection with histidine, the work of Windaus and Knoop on the formation of methylimidazole from glucose must be mentioned on account of the possible synthesis in the animal body of both histidine and purine bases.

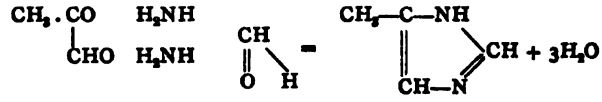
It has long been known that glucose is converted by alkalies into lactic and other oxy acids. Knoop and Windaus investigated this reaction in the presence of ammonia, in the form of the strongly dissociated zinc hydroxide ammonia. By exposing glucose and zinc hydroxide ammonia to diffused daylight they obtained methylimidazole. Inouye has also obtained this compound employing galactose and arabinose instead of glucose.

The formation of methylimidazole is explained as follows :—

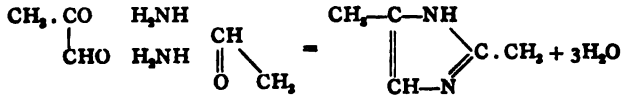
From the sugar glyceric aldehyde is probably first formed, and this is converted into methyl glyoxal by loss of water, from which lactic acid may arise by the subsequent addition of water :—

$$CH_2OH.CHOH.CHO \rightarrow CH_3.CO.CHO \rightarrow CH_3.CHOH.COOH$$

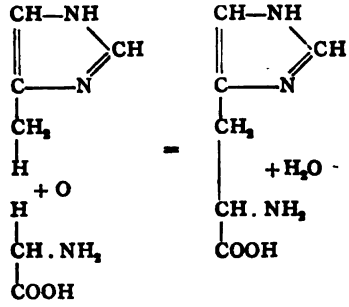
In the presence of ammonia and formaldehyde, also a product from the sugar, methylimidazole is formed as follows:—



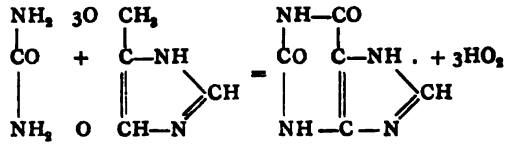
This condensation with formaldehyde as well as with methyl glyoxal is confirmed by the formation of dimethylimidazole when ammonia acts upon glucose and acetaldehyde.



From imidazole by condensation with glycocoll and simultaneous oxidation histidine may possibly be formed thus:—

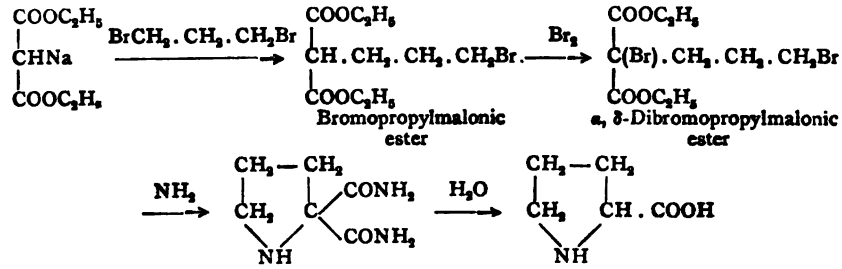


and by condensation with urea xanthine may arise:—

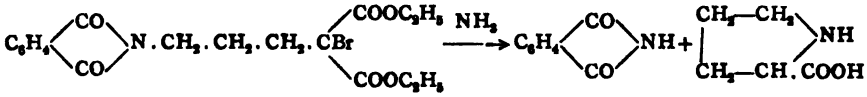


Proline.

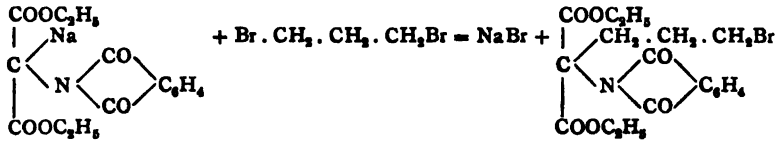
Just a year before E. Fischer obtained this compound by the hydrolysis of caseinogen, it was synthesised by Willstätter in 1900 from sodium malonic ester and trimethylene bromide by the following reactions :—



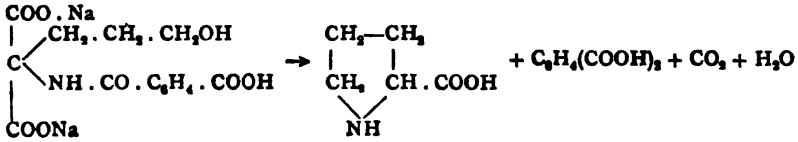
It was also synthesised by E. Fischer in 1901 from γ-phthalimidopropylmalonic ester which he employed in the preparation of ornithine. The bromine derivative of this compound when treated with ammonia gave a complex mixture of products, which, after hydrolysis by hydrochloric acid at 100° C., gave phthalimide and *a*-pyrrolidine carboxylic acid :—



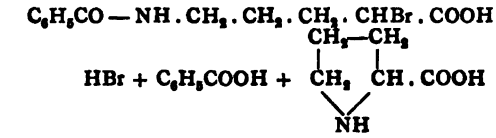
Sörensen and Andersen in 1908 synthesised proline by the sodium phthalimidomalonic ester method, a yield of about 80 per cent. being obtained. Sodium phthalimidomalonic ester is condensed with trimethylene bromide,



the resulting γ-bromopropyl-phthalimidomalonic ester is heated in alcoholic solution with sodium hydroxide and the product so formed is evaporated with hydrochloric acid. Proline is obtained instead of the expected *a*-amino-δ-ethoxyvalerianic acid, ring formation occurring just as in the other methods of preparing proline :—



Fischer and Zemplen have also found that proline is formed by boiling benzoyl-δ-amino-*a*-bromovalerianic acid (p. 119) with concentrated hydrochloric acid :—

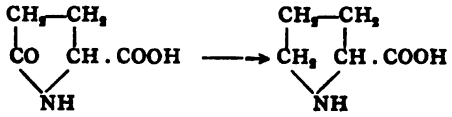


There was no difficulty in identifying the natural substance with the synthetical one, and its presence in egg-albumin, gelatin and other proteins was soon afterwards established.

The question at once arose whether this *a*-pyrrolidine carboxylic acid, or *a*-proline as Fischer termed it in 1904, was a primary product, or a secondary product formed by the action of mineral acids upon other products, but its formation by hydrolysis by alkali and by the action of pepsin followed by trypsin pointed to its being a primary product and therefore one of the units of the protein molecule. Still further proof of the primary nature of proline was given by Fischer and London in 1911. By a prolonged digestion of gliadin they obtained almost as much proline as was obtained from this protein by hydrolysis.

Sörensen, in 1905, suggested that it might arise from *a*-amino-δ-oxyvalerianic acid which he synthesised, but the fact that this amino acid has not yet been obtained by hydrolysis of protein and the above facts seem to exclude this possibility. Fischer and Boehner also obtained a greater amount of proline by hydrolyses with baryta than is usually obtained by acid hydrolysis. Its isolation, under these conditions, excludes Sörensen's amino-oxyvalerianic acid as a primary product.

Pyrrolidone carboxylic acid is readily made from glutamic acid (p. 116). Proline can be obtained, by reduction, from this compound as Fischer and Boehner showed in 1911 :—

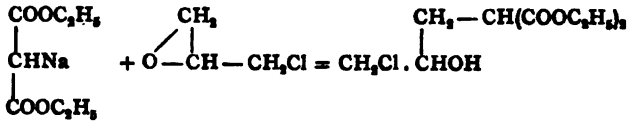


This conversion of glutamic acid into proline is of interest as it is possible that proline may be formed in nature in this manner.

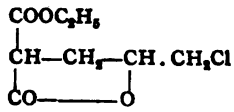
Oxyproline.

In 1902 E. Fischer isolated a compound of the empirical formula $C_5H_9O_3N$ from the hydrolysis products of gelatin. From its composition he supposed that it was an oxy-derivative of pyrrolidone carboxylic acid, and this was proved by its reduction to proline with hydriodic acid.

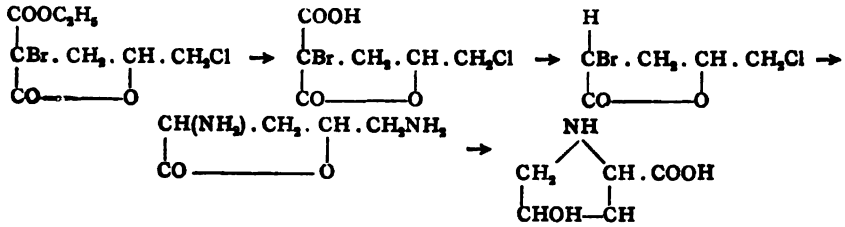
Leuchs, in 1905, synthesised two stereoisomeric-γ-oxy-prolines, one of which is probably the inactive form of the natural oxyproline. Epichlorhydrin and sodium malonic ester yield γ-chlor-β-oxy-propyl-malonic ester,



which loses alcohol and is converted into its lactone, *i.e.*, the ester of δ-chlor-γ-valerolactone-α-carboxylic acid,



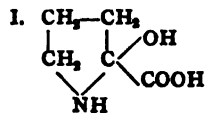
This compound on bromination, followed by hydrolysis of the ester group with hydrobromic acid and removal of carbon dioxide, gave α-brom-δ-chlor-γ-valerolactone, from which by treatment with ammonia γ-oxyproline was obtained:—

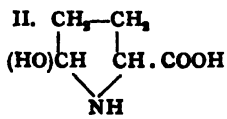


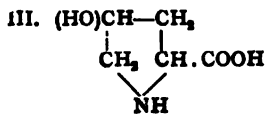
As this compound contains two asymmetric carbon atoms, four stereoisomeric forms are possible; by synthesis these must occur in two inactive forms. These forms Leuchs separated by crystallisation of the copper salts, the more insoluble acid being termed (a)-γ-oxyproline, the other (b)-γ-oxyproline.

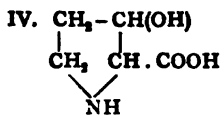
The constitution of these acids was confirmed in 1908 by Leuchs and Felser, who converted them, by reduction with hydriodic acid, into proline. Their attempt to determine whether natural oxyproline was

the active form of one of the synthetical compounds by converting the natural substance into its racemic form by heating with baryta to 200° C. was unsuccessful, since complete racemisation did not occur. As, however, all compounds containing one asymmetric carbon atom to which a carboxyl group is attached are easily racemised, the result led to the conclusion that oxyproline contains two asymmetric carbon atoms. Of the four possible formulæ,









formula I. is therefore excluded; and formula II. is not possible on account of the great stability of the acid to baryta; consequently the natural product can only be a γ- or a β-oxyproline.

Tryptophane.

The isolation of tryptophane by Hopkins and Cole in 1902 from the mixture of products formed in the tryptic digestion of caseinogen by precipitation in sulphuric acid solution with mercuric sulphate, besides adding to our list of foundation-stones or units of the protein molecule, gave us the explanation of three phenomena long known in connection with the chemistry of the proteins; namely (1) of the reddish-violet colour produced when chlorine or bromine water is added to a tryptic digest; (2) of the Adamkiewicz reaction; (3) of the origin of indole, skatole and related substances occurring in putrefaction.

The first of these phenomena was described in 1826 by Tiedemann and Gmelin. They observed that a reddish-violet colour was produced on adding chlorine water to an extract of dog's pancreas. Cl. Bernard, in 1856, showed that this reaction was given by a trypsin digest of caseinogen, and Kühne, in 1875, found that bromine water gave a better reaction than chlorine water, whilst iodine did not produce the colour. Kühne showed also that this reaction was given by a pure trypsin digest in presence of chloroform, *i.e.*, without the intervention of micro-organisms, and was, in fact, the first to point out the difference between soluble ferments or enzymes, as he called them, and living ferments or bacteria. Stadelmann called the then unknown substance proteino-chromogen and the coloured body proteinochrome, whereas Neumeister, who showed that the reaction was obtained with any deep-seated decomposition of protein, whether by trypsin, baryta water or dilute sulphuric acid, gave the substance the name of tryptophane. This name Hopkins and Cole gave to their crystalline substance as it gave this reaction; its presence in the digest causes the reaction.

Shortly before Hopkins and Cole isolated tryptophane, they studied the Adamkiewicz reaction—the production of a violet colour when concentrated sulphuric acid is added to a protein dissolved in glacial acetic acid—and found that it was caused by the presence of glyoxylic acid in the glacial acetic acid, from which it arose by the action of sunlight. On applying the glyoxylic reaction to tryptophane a very intense colour was produced, and hence the presence of tryptophane in the protein molecule is the cause of this reaction.

According to Cole, the Liebermann reaction—an intense blue colour when proteins are precipitated by alcohol and washed with ether and then heated with concentrated hydrochloric acid—is also due to the presence of tryptophane in the protein and to glyoxylic acid in the

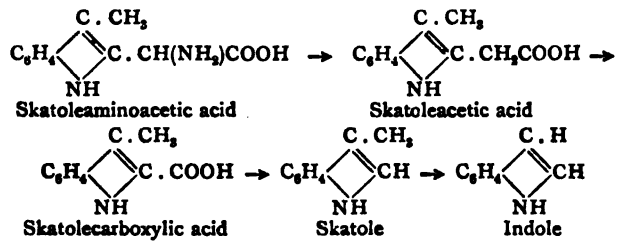
ether employed in washing the precipitated protein. The reddish-violet colour produced when proteins are heated with concentrated hydrochloric acid is due to tryptophane and to furfural formed from carbohydrate in the protein; it is very marked when cane sugar or furfural is added to a protein which does not give the reaction very strongly.

Reichl's reaction again—a green to blue colour when proteins are treated with an aldehyde such as benzaldehyde, a drop of ferric chloride and concentrated hydrochloric acid—is due also to the presence of tryptophane in the protein. Heimrod and Levene have tested a large number of aldehydes and have obtained positive results; Rohde, Steensma and Bardachzi have tested the colour reaction with aromatic aldehydes; Ekenstein and Blanksma with various furfuraldehydes.

It would appear from Osborne and Harris' results with the vegetable proteins that the production of a violet colour requires the presence of carbohydrate for the formation of furfuraldehyde.

The reaction with formaldehyde is used as a means of detecting formaldehyde in milk; its cause was also explained by Hopkins and Cole's discovery of tryptophane. Rosenheim at one time thought that the Adamkiewicz reaction was due to formaldehyde and an oxidising agent in the sulphuric acid, but Dakin has shown definitely that the reaction is due to glyoxylic acid.

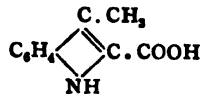
The formation of indole by the putrefaction of proteins was observed by Kühne and by Nencki in 1874, that of skatole by Brieger in 1877, of skatolecarboxylic acid by E. and H. Salkowski in 1880, and of skatoleacetic acid by Nencki in 1889. Nencki regarded these substances as originating from skatoleaminoacetic acid in the protein in a manner similar to that by which phenol, cresol, oxyphenylacetic acid and oxyphenylpropionic acid originated from tyrosine, namely:—



Tryptophane was found by Hopkins and Cole to have the empirical formula $C_{11}H_{12}N_2O_2$ and to yield large amounts of indole and skatole when heated, and when subjected to putrefaction by bacteria the above-

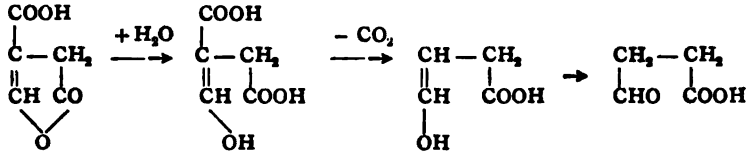
mentioned four products resulted. As under anaërobic conditions a large yield of skatoleacetic acid was obtained, and as skatole was the principal product when it was fused with potash, Hopkins and Cole regarded their substance as skatoleaminoacetic acid rather than the isomeric indoleaminopropionic acid.

The constitution of indole and skatole had been proved by synthesis, but that of the other two compounds had not been determined, and Nencki's formulæ for them were accepted. Investigations by Ellinger and Gentzen in 1903, who found that in the large intestine indole was formed in large amounts from tryptophane, but skatole only in small amounts, and that skatole only gave traces of indole under the same conditions led Ellinger to doubt the accuracy of Nencki's formulæ for skatoleacetic and skatolecarboxylic acids, more especially as Wislicenus and Arnold's skatolecarboxylic acid, which was synthesised from propionyl formic acid phenylhydrazone had the formula

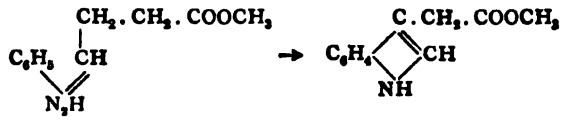


and was not identical with the putrefaction product, which might equally well possess the constitution of

The synthesis of this compound by Ellinger from β-aldehydopropionic acid, prepared from aconic acid as follows:—

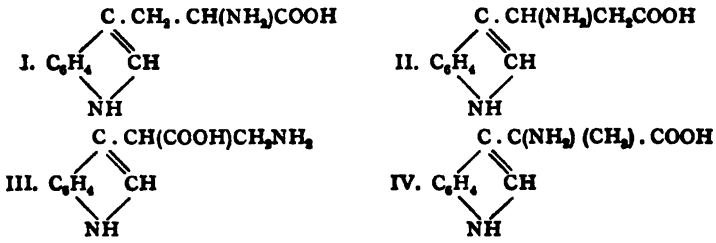


by condensation with phenylhydrazine, and the subsequent treatment of the ester of the hydrazone which was formed with alcoholic sulphuric acid,

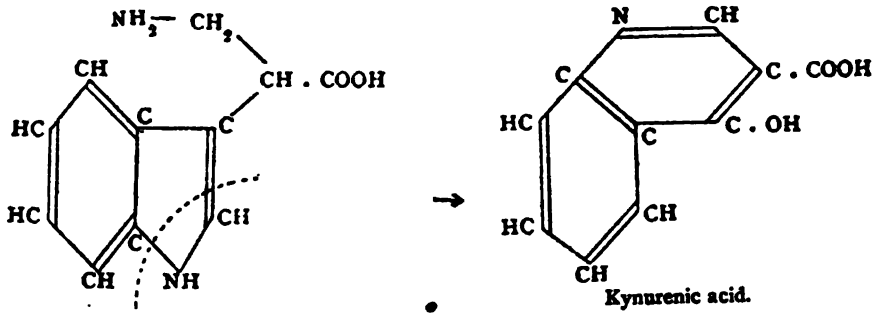


showed that it was identical with Salkowski's acid, and that it was indoleacetic acid and not skatolecarboxylic acid.

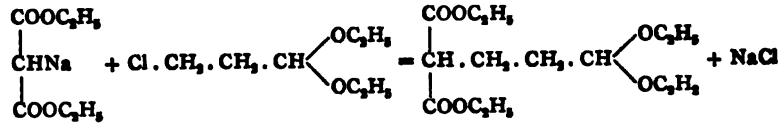
Four formulæ were therefore possible for tryptophane :—



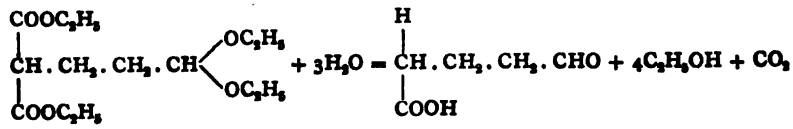
and as Ellinger had found that tryptophane when given to dogs and rabbits was converted into kynurenic acid, formula III. was regarded as the most probable for tryptophane, because it most easily explained this change :—



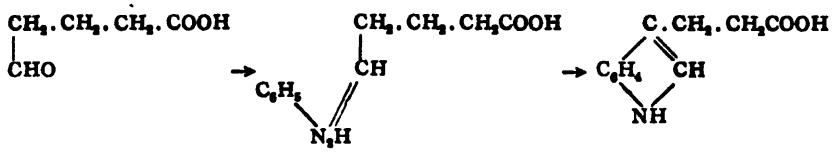
Ellinger's further work did not confirm this supposition. By condensing β-chloropropionacetal with sodium malonic ester he obtained propionacetal malonic ester,



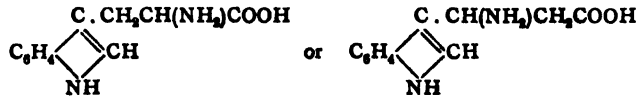
which, when heated in a sealed tube with water to 190°, was converted into γ-aldehydobutyric acid with loss of alcohol and carbon dioxide :—



The hydrazone of this compound when treated with alcoholic sulphuric acid gave the ester of indolepropionic acid :—



The indolepropionic acid obtained by hydrolysis was identical with Nencki's skatoleacetic acid, and tryptophane was therefore either

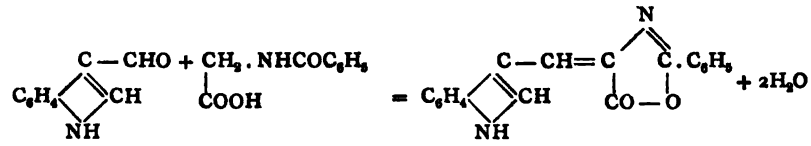


and kynurenic acid must be formed by some other reaction.

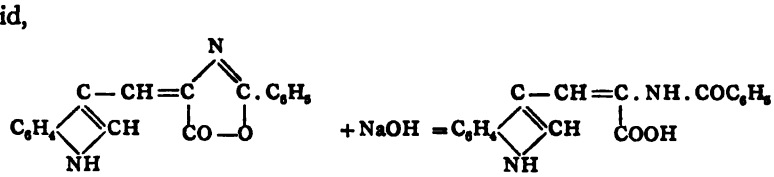
Hopkins and Cole had obtained by the oxidation of tryptophane with ferric chloride a body of the composition of C_9H_7NO; this body has been shown by Ellinger to be β-indole-aldehyde, firstly by oxidising it to β-indole-carboxylic acid,

a compound synthesised by Ciamician and Zatti, and secondly by synthesis from indole and alcoholic chloroform. From this compound by the method employed by Erlenmeyer in the synthesis of phenylalanine, Ellinger and Flamand synthesised tryptophane in 1907:—

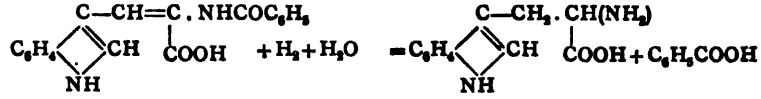
) By condensing β-indolealdehyde with hippuric acid the azlactone is obtained,



which, when boiled with dilute soda, gives indolyl-α-benzoylaminoacrylic acid,



This compound on reduction with sodium amalgam and hydrolysis with water gives tryptophane:—



E. The Optically Active Amino Acids.

1. Separation of Racemic Mixture.

With the exception of glycine, all the amino acids contain an asymmetric carbon atom, and they are therefore capable of existence in two optically active forms. In one of these forms they are present in the protein molecule, and the synthesis of a naturally occurring amino acid is only completed when the synthesised compound has been separated into its optically active components.

Three methods are known, all due to Pasteur, by which an inactive mixture can be separated into its optically active isomers:—

1. The crystallisation and mechanical separation of the two isomers.

2. The action of micro-organisms—moulds, yeasts—which destroy the one isomer more rapidly than the other. This is known as the biological method.

3. The fractional crystallisation of the salts of these compounds with optically active bases or acids.

By all these methods the optically active forms of the amino acids have been prepared.

By the first method Piutti, in 1887, obtained dextro- and lævo-aspartic acid. He re-crystallised natural asparagine and separated it into dextro- and lævo-asparagine, from which compounds he prepared both the dextro- and the lævo-aspartic acids. As, however, Piutti used asparagine from vetch seedlings, this separation cannot strictly be said to be the actual synthesis of a natural compound, but there seems no reason to doubt the possibility of separating synthetical asparagine by this means.

Glutamic acid, the homologue of aspartic acid, according to Menozzi and Appiani, on recrystallisation from water, can be obtained in its two enantiomorphous forms.

By the second method Schulze and Bosshard prepared d-leucine and l-glutamic acid, and Engel prepared d-aspartic acid. Menozzi and Appiani also separated glutamic acid by this method. In all these cases the mould *Penicillium glaucum* was used to effect the separation.

From inactive cystine Neuberg and Mayer separated d-cystine, using *Aspergillus niger* instead of *Penicillium glaucum*, which gave no result with this amino acid. Fischer found that *Aspergillus niger*, when cultivated on alanine, destroyed 10 per cent. of the natural isomer.

Not only moulds, but also yeasts can be employed in the separation of optically active compounds as was shown by F. Ehrlich in 1906, who obtained in this way l-alanine, d-leucine, l-valine. Further, amino acids, other than those which occur in nature, can be separated by moulds and yeasts into their components, *e.g.*, n-*α*-aminocaproic acid, methylethylaminoacetic acid.

It was first shown by E. Fischer, in 1894, that enzymes were specific in their action; thus maltase acts only upon *α*-glucosides and emulsin only upon *β*-glucosides. Later, he found that trypsin acted "asymmetrically" upon inactive polypeptides, *e.g.*, alanyl-leucine was hydrolysed in such a way that only the compound composed of d-alanine and l-leucine, the natural isomers, was split up into its constituents, whereas the compound composed of l-alanine and d-leucine was unattacked. Again, inactive leucine ester was found by Warburg to be only partially hydrolysed by trypsin; he obtained l-leucine and d-leucine ester.

Kossel and Dakin, in 1904, found that d-arginine was hydrolysed by the enzyme arginase into d-ornithine and urea; and by using this enzyme Riesser, in 1906, separated dl-arginine, which he had prepared by heating d-arginine with sulphuric acid to 160-180° C. into l-arginine, d-ornithine and urea, the racemic compound being hydrolysed asymmetrically by the enzyme. l-Ornithine can be prepared from the l-arginine by hydrolysis with baryta.

Bacteria can also produce a separation of the stereoisomers. Neuberg and Karczag found that the natural isomer of valine was attacked, leaving the unnatural isomer.

The biological method thus only serves for the preparation of that isomer which does not occur in nature, since the mould or yeast or enzyme destroys the naturally occurring form, leaving the other isomer untouched. According to Marckwald and Mackenzie both isomers are attacked, the natural one more rapidly than the other. This has also been shown by Pringsheim in the cases of leucine and glutamic acid.

The method therefore does not lead to the synthesis of the naturally occurring amino acid.

The third method of separating optically active substances by combining them with optically active bases, or acids, had not been employed with any success with the amino acids until E. Fischer took up this question, the study of the optically active amino acids being his first work upon the chemical constitution of the proteins. The non-success of this method was in all probability due to the small affinity which the simple amino acids themselves have for combining with acids and bases; even the attempts to separate the monoaminodicarboxylic acids, which are fairly strong acids, were not successful.

Hippuric acid, or benzoylglycine, has been known for a long time, and by preparing the benzoyl derivatives of the other amino acids, Fischer found that their acidic character was greatly increased, and that they then combined with the optically active bases brucine, strychnine, cinchonine, morphine, forming stable salts. These salts were much less soluble and their power of crystallising much greater than the salts of the amino acids themselves, and consequently they were more easily isolated; further, they were easily reconverted into the amino acids.

These benzoyl derivatives were prepared by shaking the amino acid with excess of benzoyl chloride in the presence of sodium bicarbonate instead of in the presence of excess of alkali, *i.e.*, by the Schotten-Baumann method, which gave poor and varying yields of the benzoyl compound.

Alanine, aspartic acid, glutamic acid, tyrosine, leucine, phenylalanine and also α-amino-n-caproic acid and α-aminobutyric acid have in this way been separated by Fischer into their optically active isomers. To these must be added ornithine which was synthesised by Sörensen in 1903, and separated into d- and l-ornithine in 1905.

Not only can the benzoyl derivative be employed for this purpose but also the formyl derivative which is prepared by heating the amino acid with anhydrous formic acid at 100° C. These formyl derivatives also give beautifully crystalline salts with the optically active bases, and they possess one great advantage over the benzoyl derivatives, namely, that the formyl group is easily removed by hydrolysis, whereas the benzoyl group requires prolonged heating with a large excess of acid for its removal. The formyl derivative is of enormous advantage also for building up optically active polypeptides, as it admits of the preparation of large quantities of the optically active amino acids.

Fischer and his pupils have thus prepared the optically active forms of leucine, phenylalanine, and valine, and also of phenylaminoacetic acid, α-amino-n-caproic acid and β-amino-β-phenyl propionic acid

and Locquin has prepared, by means of the formyl derivative, d-isoleucine.

In the case of serine, no separation could be effected by means of these derivatives, but, by making the p-nitrobenzoyl compound, Fischer and Jacobs obtained d- and l-serine.

Proline was separated in 1909 by Fischer and Zemplen by the fractional crystallisation of the cinchonine salt of its m-nitrobenzoyl compound. This was prepared from m-nitrobenzoyl-δ-amino-valerianic acid by the same reaction as was employed in its synthesis (p. 131).

Isoserine and diaminopropionic acid must also be added to the list of optically active amino acids separated by Fischer and his pupils.

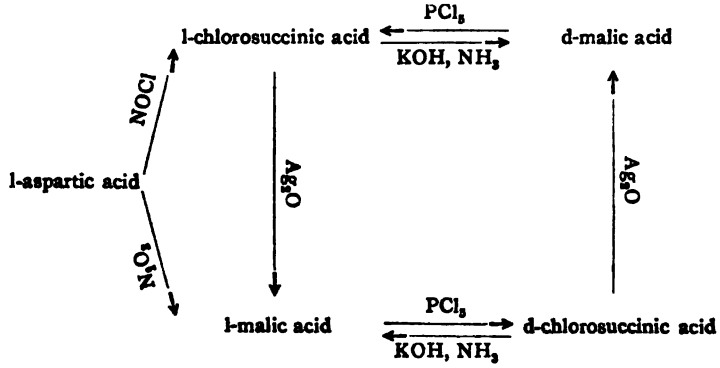
In all these cases the acidic function of an amino acid has been made use of for purposes of separation. The first case in which the basic function of an amino acid was requisitioned was that of Betti and Mayer in 1908, who separated α-aminophenylacetic acid into its isomers by combining it with d-camphorsulphonic acid. Fischer and Scheibler, in 1911, found that this was the only means of obtaining the isomers of β-aminobutyric acid.

Pyman, in 1911, used the basic function of histidine to obtain the natural isomer; the racemic compound was easily separated by the fractional crystallisation of its salt with d-tartaric acid.

The specific rotatory powers of the natural and synthetical amino acids are given in Table B, p. 149.

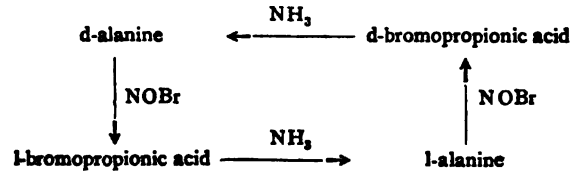
2. The Walden Inversion.

It was first observed by Walden, in 1896, that a change of configuration took place in the conversion of the malic acids into the chlorosuccinic acids and *vice versâ*. His results were collected together in 1897 and expressed in the following scheme, in which was included Tilden and Marshall's observations on aspartic acid :—



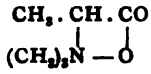
Walden concluded that potassium hydrate and phosphorus pentachloride acted optically normally, *i.e.*, without alteration of the configuration, but that silver oxide, and therefore also nitrous acid and nitrosyl chloride, acted optically abnormally, but which of these reactions was really the normal one he was not able to decide. The conclusion was remarkable, since the action of silver oxide takes place in aqueous solutions at a low temperature and the effect of potash in producing racemisation is well known. Still more curious is the supposition that nitrous acid and nitrosyl chloride act optically abnormally.

A similar change in rotation was observed in 1905 by Fischer and Warburg in the conversion of alanine into the corresponding halogen fatty acid by nitrosyl bromide and in the reconversion of this compound into alanine by the action of ammonia :—



This was termed the "Walden inversion" by Fischer in 1907.

A change in configuration occurs either by the action of ammonia, or by the action of nitrosyl bromide. By studying the conversion by the action of ammonia under various conditions, Fischer was able to show conclusively that this reagent behaved optically normally, which result was confirmed by a later experiment upon optically active trimethyl-α-propiobetaine (α-homobetaine).



which he prepared from trimethylamine and d-α-bromopropionic acid, and showed it to be identical with that prepared by the action of methyl iodide upon d-alanine.

The change produced by nitrosyl bromide on d-alanine was found to be optically abnormal; the following reactions occurred:—

$$\text{d-alanine} \xrightarrow{NOBr} \text{l-bromopropionic acid}$$

$$\text{d-alanine ester} \xrightarrow{NOBr} \text{d-bromopropionic acid ester}$$

which were confirmed by similar observations upon l-leucine ester, l-phenylalanine ester and on l-aspartic ester. The same reagent thus acts optically normally on the ester and optically abnormally upon the acid.

Silver oxide behaves like nitrosyl bromide; it acts normally on a derivative, but abnormally on the acid. These changes

$$\text{l-bromopropionic acid} \xrightarrow{Ag_2O} \text{d-lactic acid}$$

$$\text{l-bromopropionyl-glycine} \xrightarrow[\text{and hydrolysis}]{Ag_2O} \text{l-lactic acid}$$

$$\text{l-bromopropionic acid} \xrightarrow{KOH} \text{l-lactic acid}$$

were observed.

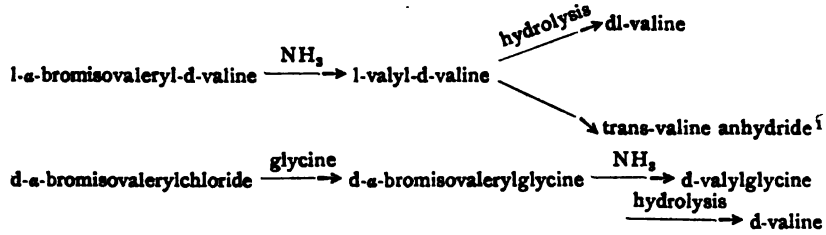
All amino acids would be expected to behave in the same way as leucine, etc., but valine was found by Fischer and Scheibler in 1908 to behave differently:—

$$\text{l-valine} \xrightarrow{NOBr} \text{d-}\alpha\text{-bromisovalerianic acid} \xrightarrow{NH_3} \text{l-valine}$$

$$\text{d-valine} \xrightarrow{NOBr} \text{l-bromisovalerianic acid} \xrightarrow{NH_3} \text{d-valine}$$

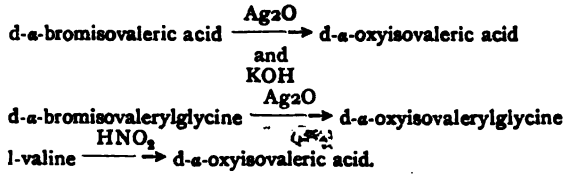
that is, the same valine and not its optical antipode was obtained.

It seemed very improbable that a "Walden inversion" had occurred twice, but the further experiments proved that this had actually happened:—

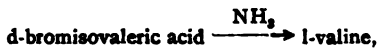


The protection of the carboxyl group has again prevented the inversion; the experiments on the interconversion of valine ester and bromisovalerianic ester were not successful.

The changes of the bromisovaleric acid and of valine into the oxy-acid were also different from those observed with the other acids:—



Here nitrous acid acts abnormally, but silver oxide acts normally. The change,



is also abnormal.

The difference between valine and the other amino acids may be due to the effect of the isopropyl group. In valine,

$$(CH_3)_2 . CH . CH(NH_2) . COOH,$$

it is attached directly to the asymmetric carbon atom; in leucine,

$$(CH_3)_2 . CH . CH_2 . CH(NH_2) . COOH,$$

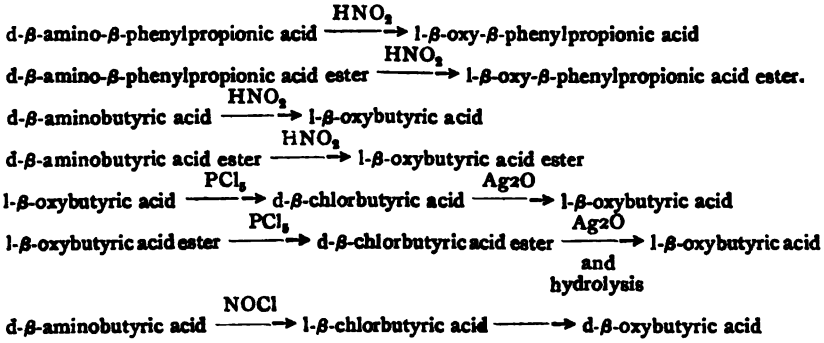
a methylene group is present between it and the $CH(NH_2)$ group which contains the asymmetric carbon atom.

If phosphorus pentachloride ammonia and potash act normally since the products have the same configuration, it may be concluded that the "Walden inversion" is limited to the reactions between nitrosyl bromide and the amino group and between silver oxide and halogen fatty acid, and that it is dependent upon the presence of the carboxyl group.

In order to ascertain if the Walden inversion, caused by the action of nitrosyl bromide and silver oxide, were due to the attachment of the carboxyl group to the asymmetric carbon atom, Fischer and Scheibler

[1] See Part II. for these compounds.

and Groh have performed experiments with β-amino acids and β-oxy acids and have observed :—


d-β-amino-β-phenylpropionic acid HNO2 l-β-oxy-β-phenylpropionic acid
d-β-amino-β-phenylpropionic acid ester HNO2 l-β-oxy-β-phenylpropionic acid ester.
d-β-aminobutyric acid HNO2 l-β-oxybutyric acid
d-β-aminobutyric acid ester HNO2 l-β-oxybutyric acid ester
l-β-oxybutyric acid PCl5 d-β-chlorbutyric acid Ag2O l-β-oxybutyric acid
l-β-oxybutyric acid ester PCl5 d-β-chlorbutyric acid ester Ag2O and hydrolysis l-β-oxybutyric acid
d-β-aminobutyric acid NOCl l-β-chlorbutyric acid d-β-oxybutyric acid

No change occurs with nitrous acid nor with phosphorus pentachloride, as the same product is obtained both with the ester and the free acid; d-β-aminobutyric acid has therefore the same configuration as l-β-oxybutyric acid. Since d-β-oxybutyric acid is formed from d-β-aminobutyric acid a Walden inversion has been produced by the action of nitrosylchloride. The result is the same as with the α-amino acids.

Reviewing the data in 1911 Fischer stated that he thought that the "Walden inversion" was not of the nature of a rearrangement of the groups attached to the carbon atom, but that it was simply the general and normal process of substitution which could be observed on account of the asymmetry of the carbon atom in these cases.

Adopting Werner's view that it was not necessary for the new group to take up the same position as the old group but that it may take up a different position, and regarding the process of substitution as if it were preceded by the formation of complex molecules, as Kekulé suggested, then during the reaction of the complex molecule the new group may take up a different position to that held by the old group. If in the reaction the shifting take place in every molecule or does not take place at all, then the new compound will have only one configuration; if, however, there be only a partial shifting, then a mixture will result. In the case of optically active compounds we shall have either the one isomer, or the other, or a mixture which represents the racemic form. As racemisation always occurs in the "Walden inversion" this hypothesis gives a good explanation of the observed phenomena. The reaction is very easy to follow with a model such as Fischer used to represent his views, but they may also be represented on a plane surface.

If we picture a carbon atom with its four valencies A, B, C, D, as a sphere (1) from which the four groups project, then a reacting molecule may attach itself at any vacant place between them (2). If the reaction take place between B and X, at the moment of reaction B will become

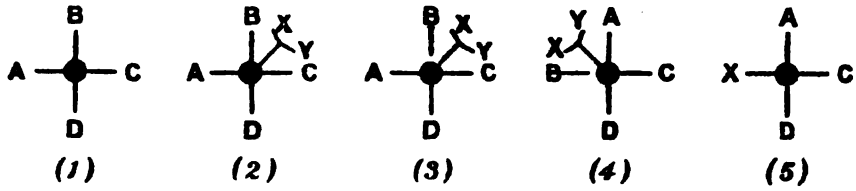


FIG. 6.

loosened (3). At this moment A or C or D may, on account of the reaction, alter their position and take the place of B. The new group must then enter the position of A, or C, or D. The compound (5) will result, if the shifting take place in every molecule.

The same hypothesis explains the reactions which occur with unsaturated compounds, but the deductions are more difficult to follow. It also will explain substitution in compounds with several asymmetric carbon atoms; if a homogeneous product result, then there is normal substitution or complete inversion; if a mixture of products which are not optical antipodes, then there is partial inversion or complete racemisation of the asymmetric carbon atom affected.

3. The Correlation of the Configurations of the Amino Acids.

Since in the interconversion of the amino-, chloro- or bromo-, and hydroxy-acids a "Walden inversion" of the groups attached to the asymmetric carbon atom occurs it is not yet possible to correlate the configuration of the amino acids together nor with that of other optically active compounds, especially the tartaric acids and glucose.

The configuration of d-malic acid is known with reference to d-tartaric acid and d-glucose.

Since no "Walden inversion" occurs when amino acids are converted into the corresponding hydroxy acid by the action of nitrous acid, the configuration of d-aspartic acid, which gives d-malic acid, may be said to be established.

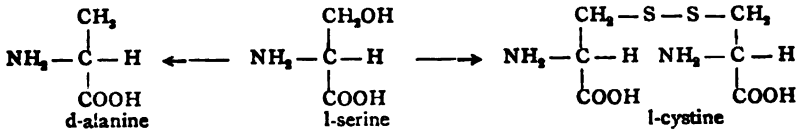
A glyceric acid results by the action of nitrous acid on serine. Neuberg and Silbermann believed that they obtained l-glyceric acid on account of the relationship of this acid to l-tartaric acid, but, a little later, Neuberg stated that the observation required confirmation.

The correlation of the configurations of l-serine, d-alanine and l-cystine was established in 1908 by Fischer and Raske by means of l-α-amino-β-chloropropionic acid,

$$CH_2Cl \cdot CH(NH_2) \cdot COOH,$$

which they obtained from l-serine methyl ester by the action of phosphorus pentachloride and subsequent hydrolysis with hydrochloric acid. By reducing it with sodium amalgam they obtained d-alanine, and by treating it with barium hydrosulphide and oxidising the resulting cysteine, by drawing a current of air through the solution, they obtained cystine.

If l-serine has the configuration represented, d-alanine and l-cystine will have the groups attached to the asymmetric atom arranged in the same order as in l-serine :—



The foundation for the configuration of these amino acids will be obtained if α-amino-β-chloropropionic acid can be converted into aspartic acid, the configuration of which is known from its relationship to malic acid.

Fischer and Flatau have commenced this synthesis by preparing methylsuccinic acid from d-alanine :—

d-alanine → l-α-bromopropionic acid → l-methylsuccinic acid.

If no "Walden inversion" occurs the l-bromopropionic acid and l-methylsuccinic acid will have the same configuration.

Kay has shown a correlation between methylisoserine and bromomethyl-lactic acid :—

$$\text{d-}\alpha\text{-methylisoserine} \xrightarrow{NOBr} \text{d-}\beta\text{-bromo-}\alpha\text{-methyl-lactic acid.}$$

TABLE B1.

SPECIFIC ROTATORY POWER OF THE NATURAL AMINO ACIDS.

	In Water	In Alkali	In HCl	Observed by
d-Alanine	+ 2·7°	...	+ 10·3°	Fischer
d-Valine	...	...	+ 27·9°	Schulze and Winterstein
l-Leucine	...	10 % + 6·65°	+ 18·9°	,, ,,
,,	− 10·4°	4 % + 8·05°	20 % + 15·7°	Ehrlich
d-Isoleucine	+ 9·7°	$\frac{N}{1}$ + 11·09°	,, + 36·8°	,,
l-Phenylalanine	− 35·3°	...	...	Schulze
l-Tyrosine	...	...	21 % − 8·5°	Schulze and Bosshard
,,	...	...	4 % − 15·6°	,, ,,
,,	...	...	,, − 13·2°	Fischer
l-Serine	...	...	+ 11·6°	,,
l-Cystine	...	− 97·5°	− 223 to − 224·3°	Mörner
l-Aspartic Acid	...	− 2·4°	+ 25·7°	Fischer
d-Glutamic Acid	+ 24·0°	...	+ 30·8°	,,
,,	...	...	+ 31·1°	Schulze and Bosshard
d-Ornithine	...	...	...	...
d-Arginine	...	...	+ 21·2°	Gulewitsch
d-Lysine	...	...	+ 17·5°	Lawrow
l-Histidine	− 39·7°	...	+ 6·5°	Kossel
l-Proline	− 77·4°	− 83·5°	− 46·5°	Fischer
l-Oxyproline	− 81·1°	...	...	,,
l-Tryptophane	− 33°	...	...	Hopkins and Cole
,,	− 30° to − 40°	$\frac{N}{1}$ + 5·7°	− 13·5°	Fischer
,,	− 30·3°	$\frac{N}{2}$ + 6·2°	+ 1·3°	Abderhalden and Baumann

TABLE B2.

SPECIFIC ROTATORY POWER OF THE SYNTHETICAL AMINO ACIDS.

	In Water	In Alkali	In HCl	Observed by
d-Alanine	...	...	+ 9·7°	Fischer
l-Alanine	...	...	− 10·3°	,,
d-Valine	+ 6·4°	...	20 % + 28·7°	,,
l-Valine	...	...	,, − 28·7°	,,
,,	− 6·1°	...	,, − 27·4°	Ehrlich and Wendel
d-Leucine	...	+ 10·4°	,, − 15·6°	Fischer, Warburg and Ehrlich
l-Leucine	...	...	,, + 15·8°	,, ,, ,,
d-Isoleucine	+ 11·3°	...	,, + 40·6°	Locquin
l-Isoleucine	− 10·6°	...	,, − 31·4°	,,
d-Phenylalanine	+ 35·1°	...	18 % + 7·1°	Fischer and Mouneyrat
l-Phenylalanine	...	...	...	...
d-Tyrosine	...	...	21 % + 8·6°	Fischer
l-Tyrosine	...	...	,, − 8·6°	,,
,,	...	...	4 % − 13·2°	,,
d-Serine	+ 6·9°	...	− 14·3°	Fischer and Jacobs
l-Serine	− 6·8°	...	+ 14·5°	,, ,,
d-Cystine	...	...	...	...
l-Cystine	...	...	− 209·6°	Fischer and Raske
d-Aspartic Acid	...	...	− 25·5°	Fischer
l-Aspartic Acid	...	− 2·3°	...	,,
d-Glutamic Acid	...	...	+ 30·8°	,,
l-Glutamic Acid	− 12·9°	...	− 30·1°	,,
l-Histidine	− 38·1°	...	...	Pyman
d-Histidine	+ 39·3°	...	...	,,
l-Proline	− 80·9°	− 93·0°	...	Fischer and Zemplen
d-Proline	+ 81·9°	...	...	,, ,,

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